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## QUANTITATIVE DROP ANALYSIS

### XIII. THE FORMOL TITRATION OF AMINO NITROGEN\*

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In the course of certain studies of the metabolism and nitrogen utilization of animal tissues growing in *in vitro* culture, it became necessary to determine changes in amino nitrogen content of the culture medium. This material, composed of blood plasma and embryo extract or fractions of these constituents, was available only in small quantity and had a very low content of amino nitrogen, making necessary the application of drop scale methods for its analysis.

Although numerous procedures have been employed for the determination of amino nitrogen in various types of materials, relatively few have been found applicable for ultramicrodetermination of this constituent. Linderström-Lang and Holter (1) first adapted the alcohol titration method to the ultramicro scale for determination of hydrolysis of peptides of low molecular weight by thin sections of plant and animal tissues. In this type of substrate, the method was shown to be both adequate and simple. For application to biological materials of more complex composition, the formol titration originated by Sørensen (2) has proved more satisfactory on the macro scale than most other methods. In line with this, Borsook and Dubnoff (3) recently developed an ultramicroprocedure in which the formol titration was carried out electrometrically with the glass electrode, along the lines investigated earlier by Dunn and Loshakoff (4). No data were quoted for evaluation of their procedure except a claim of better than  $\pm 2$  per cent accuracy. It is well recognized (5, 2) that the

\* Aided by grants from the Rockefeller Foundation and the Research Board of the University of California.



formol titration is an empirical method which yields quite different recoveries with different amino acids and with different titration conditions. With the unknown mixtures found in biological materials, the recovery will not usually be quantitative or known, but may have considerable comparative value. Only a limited number of amino acids yield recoveries of approximately 100 per cent, a few dropping to values as low as 50 to 80 per cent. In view of these facts, it is possible that the claim of Borsook and Dubnoff refers to precision rather than accuracy, or that compensating errors in their particular material balanced the known deficiencies of recovery.

It seemed desirable to ascertain whether indicators could be used for drop scale determinations of amino nitrogen by the drop scale formol titration, and to compare the data so obtained with those obtained by the procedure of Borsook and Dubnoff. The method here described, while definitely simpler than the electro-metric titration, is shown to have a comparable accuracy and precision when tested with pure amino acids, and to agree quite well with the published data obtained by use of the macro formol titration. Its application to the analysis of embryo extract was also incompletely studied in connection with recovery of added amino acids when various deproteinizing agents were used. This work will be presented in detail in a later publication.

#### EXPERIMENTAL

##### *Reagents—*

1. *Stock amino acid solutions* were prepared by weighing the proper quantity of each to yield a 0.01 M solution, with purified and analyzed samples of amino acid and redistilled water. The amino acids used were glycine, glutamic acid, serine, lysine, histidine, and phenylalanine, all of which were highly purified samples available in the laboratory.

2. *Formaldehyde solutions* were prepared from Merck reagent grade formaldehyde, 36 to 38 per cent, diluted with redistilled water to various strengths, usually 1 volume of formaldehyde with 2 volumes of water.

3. *Indicator solution*, usually phenolphthalein in 0.03 per cent solution in 50 per cent alcohol. This was diluted with an equal volume of water before use.

*Apparatus*—The apparatus used was, for the most part, the standard drop analysis equipment,<sup>1</sup> a preliminary description of which has been published by Kirk (6). The burette was an improved form of the universal capillary type shown, respectively,

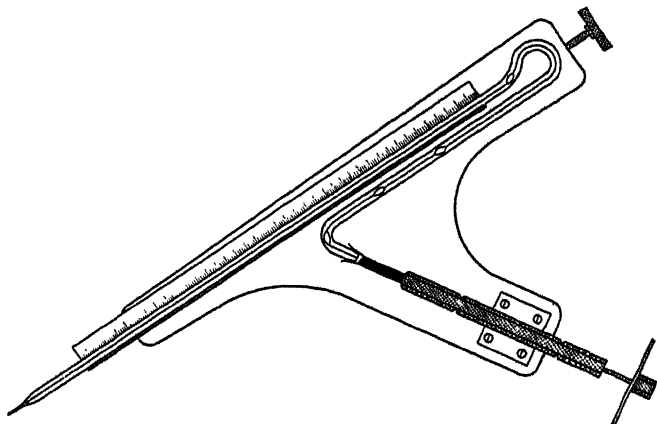


FIG. 1. Front assembly of the universal type capillary burette

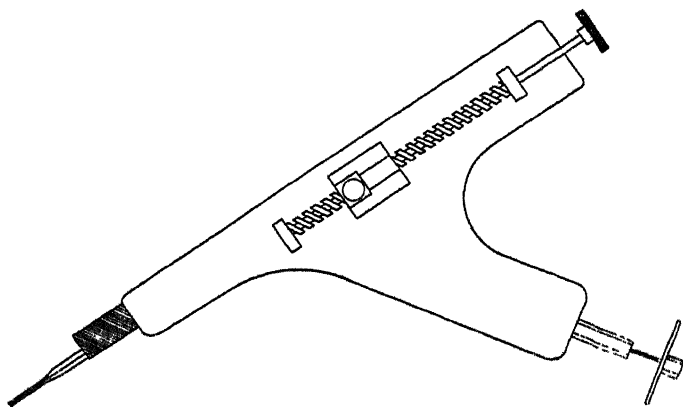


FIG. 2. Rear assembly of the universal type capillary burette

in front and rear views in Figs. 1 and 2. It had a total capacity of about 0.1 ml., and was capable of a precision in reading of  $\pm 0.03$

<sup>1</sup> All apparatus, except electrical measuring instruments, was obtained from the Microchemical Specialties Company, Berkeley.

$\lambda$ .<sup>2</sup> Samples and reagents were measured with the same precision by use of capillary pipettes calibrated with mercury for content, and washed out with a little water to avoid drainage errors. Their form and that of the pipette control are shown in

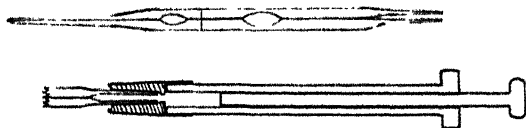


FIG. 3. Capillary pipette and syringe control

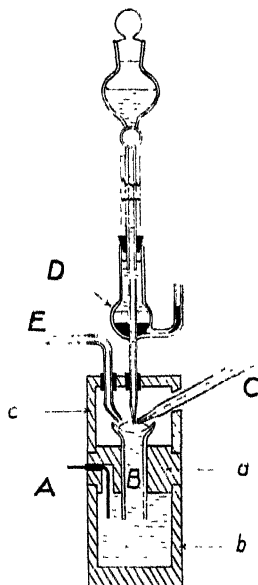


FIG. 4. Cross-section of the glass electrode titration vessel. *B* represents an inverted glass electrode; *C*, a burette; *D*, a reference calomel cell; *E*, a glass tube; *A*, cup assembly, consisting of *a*, a central block; *b*, a lower cup; *c*, an upper inverted cup.

cross-section in Fig. 3. The pipettes were constructed with a taper on the upper end so as to slip through a rubber gasket encased in a metal fitting on the end of a 0.5 ml. syringe barrel.

<sup>2</sup>  $1 \lambda = 1 \text{ microliter} = 0.001 \text{ ml. (6).}$

Electrometric titrations were performed by use of a glass electrode used in conjunction with a vacuum tube galvanometer and a Leeds and Northrup hydrogen ion potentiometer. The details of the glass electrode vessel may be seen in cross-section in Fig. 4. *B* is an inverted glass electrode constructed from Corning No. 015 glass by blowing a bulb and sucking in a depression, the latter serving as a titration vessel. All of the bulb except the depression was coated with paraffin on the outside. The bulb was filled with 0.1 *N* hydrochloric acid solution saturated with quinhydrone, inverted, and inserted into the chamber, the lower part of which had been filled with the same reference solution. The outside chamber *A* was constructed of the synthetic plastic, lucite, by use of a lathe, being made in three parts, *b* a lower cup, *a* a central block drilled as shown, and an upper inverted cup (*c*) also drilled for insertion of a reference calomel cell *D*, a burette *C*, and a glass tube *E*. Through the last, nitrogen was blown on the surface of the sample to produce a whirling action, which effectively stirred it and prevented entrance of atmospheric carbon dioxide during titration.

### *Procedure*

*Indicator Method*—A sample (about 50  $\lambda$  of 0.01 *M* amino acid solution) was carefully measured with rinsing into a porcelain titrating dish. To this a known quantity (usually about 10  $\lambda$ ) of diluted phenolphthalein solution was added, and the sample titrated to a faint pink color by use of standard (about 0.02 *N*) sodium hydroxide. This preliminary end-point was chosen because it obviated the necessity of using a second indicator. A volume of formaldehyde, diluted as stated above and equal to the volume of the sample, was then added. The mixture was again titrated to the phenolphthalein end-point while being stirred. A blank value was determined with distilled water instead of the sample to correct for indicator and formaldehyde titers. These values normally ran from 0.5 to 1.0  $\lambda$ . The volume used in the second titration of the sample was then corrected by subtraction of the corresponding blank and calculated in terms of amino nitrogen. In case of ammonia or ammonium salts being present in the sample, it is necessary to make proper analysis for their presence. Theoretically, the ammonium ion is nearly quantita-

tively determinable by use of the formol titration, but the recovery is, to a considerable extent, dependent on the particular shade of pink chosen as the end-point before addition of formaldehyde. Complete recovery of ammonia may not be obtained by the method given, and it is always advisable to run a controlled determination on known amounts of ammonia in order to make proper allowance for this factor. The independent estimation of ammonia and the application of the recovery factor properly corrected the amino nitrogen values for the ammonia present.

*Electrometric Method*—With the electrometric set-up as described, the titration was run by essentially the same method as outlined by Borsook and Dubnoff (3). In this case, the sample was measured as before and placed in the depressed cup of the glass electrode. It was adjusted to pH 7, the electrode being used to determine this point. Formaldehyde previously adjusted to pH 5 was added in equal volume, and the mixture titrated to a final pH of 8. A blank value was determined as before and subtracted from the titer of the unknown. In order to assure the reliability of the electrode, frequent checks against standard buffers were made, and a constancy to  $\pm 2$  millivolts throughout the period of titration was maintained.

### Results

*Indicator Method*—In developing a proper procedure for drop scale formol titration, it was necessary to study the various factors concerned. There are only two important variables which may cause difficulty; namely, the effect of formaldehyde concentration and the effect of the indicator chosen. The first factor was studied by making a series of determinations on 0.01 M solution of serine, with variable concentrations of formaldehyde. The data are shown in Table I. It is readily apparent that the optimum concentration was 1 volume of the formaldehyde diluted with 2 volumes of redistilled water to give a final formaldehyde concentration after titration of about 2.7 per cent. This figure is definitely lower than that used by Sørensen (2), and was somewhat lower than that of Borsook and Dubnoff (3). That this factor may vary on the drop scale from the optimum concentration when larger volumes are used is entirely possible and not contrary to experience with other reagents. In order to check the effect of the end-point chosen, thymolphthalein, methyl red, phenol red.

$\alpha$ -naphtholphthalein, phenolphthalein, and alizarin yellow were used. Of this group of indicators, phenolphthalein was found to be definitely the most favorable. Thymolphthalein has been widely used, but this indicator did not allow a clear end-point on the drop scale. The other indicators, except phenol red, gave poor end-points in the presence of formaldehyde. Phenolphthalein gave better recoveries than the latter indicator owing to its higher pH of color change. Some fading of end-point is occasionally encountered with phenolphthalein owing to absorption of carbon dioxide from the atmosphere. Appreciable error from this source was avoided by titrating reasonably rapidly to the first perceptible pink, and disregarding fading after this point. Table II shows

TABLE I

*Effect of Formaldehyde Concentration*

Sample, 50.87  $\lambda$  of 0.01 M serine solution = 7.12  $\gamma$  of N.

Formaldehyde dilution	Final formaldehyde concentration	Serine N found	Recovery
	<i>per cent</i>	$\gamma$	<i>per cent</i>
36-38% undiluted	8	4.70	66
		4.76	67
1 volume HCHO to 1 volume re- distilled water	4	5.84	82
		5.48	77
1 volume HCHO to 2 volumes re- distilled water	2.7	6.62	93
		6.83	96
1 volume HCHO to 3 volumes re- distilled water	2	6.05	85
		6.19	87

the results of the application of this simple titration method to the determination of amino nitrogen in six amino acids. These were chosen to represent all of the general types of amino acids without any effort to include all of the available ones. Recovery data indicate that, with the exception of certain neutral and dicarboxylic acids, definitely low results are to be expected. This finding is again in accordance with that of Sørensen and subsequent investigators. In general, the data agree rather closely with those of Sørensen.

In view of the rather wide use of glass electrodes as indicator instruments in the formol titration, the electrode technique described was applied to the same series of amino acids. The details of formaldehyde concentration and end-points chosen were

taken from Borsook and Dubnoff's description (3). An initial pH of 7 was used, and the formaldehyde added had been previously adjusted to pH 5. The mixture was finally titrated to pH 8, all of these values being those used by the above authors. The results of these titrations are shown in Table II.

TABLE II

*Indicator and Electrometric Formal Titrations of 0.01 M Amino Acid Solutions*

Amino acid	Indicator method				Electrometric method			
	Volume	Amino N in sample	Amino N found	Recovery	Volume	Amino N in sample	Amino N found	Recovery
	$\lambda$	$\gamma$	$\gamma$	per cent	$\lambda$	$\gamma$	$\gamma$	per cent
Glycine	50.87	7.12	7.08	99	93.90	13.14	11.62	88
			7.01	98			11.78	90
			7.09	99			11.49	89
			7.09	99			11.62	88
Glutamic acid	50.87	7.12	6.85	96	56.36	7.89	7.88	100
			7.15	101			8.08	102
	32.38	4.53	4.74	104			7.88	100
			4.74	104			7.90	101
Serine	50.87	7.12	6.56	92	95.85	13.42	13.17	98
			6.56	92			12.05	90
			6.48	91			12.73	95
			6.37	90			11.54	86
Phenylalanine	50.87	7.12	4.97	70	50.87	7.12	4.52	64
			5.12	72			4.55	64
			4.11	58			4.47	63
			5.08	71			4.57	64
Histidine	50.87	14.23	9.46	67	50.87	14.23	11.61	82
			9.56	67			11.52	81
			9.41	66			11.57	81
			9.55	67			11.64	82
Lysine	50.87	14.23	10.16	72	50.87	14.23	6.84	48
			10.60	74			7.04	49
			9.92	70			7.24	51
			10.12	71			7.32	51

In three cases, the recovery figures are equal to, or greater than, those of the indicator method. In the remainder, they are even lower. The variability between results with the same amino acid is at least as great in most instances as with the indicator procedure.

The general inferiority of the electrometric results to those of the indicator method is unquestionably not due to the use of the glass electrode, but rather to the particular pH values chosen in the titration. It is, for example, not clear why formaldehyde solutions should be adjusted to pH 5, which allows a large blank. As a general rule, a final pH of 9 is theoretically better than one of 8 as used in this study. The choice of these values by the authors quoted was undoubtedly made from empirical considerations which were correct for their particular system, but not useful for pure amino acids nor for systems of other types. In any case, it is preferable from a theoretical standpoint to employ a glass electrode because it is free of most of the criticisms which may be made of the indicator method. At the same time, the greater

TABLE III  
*Indicator Formol Titration of Ammonia*

Sample, 73.04  $\lambda$  of 0.01 M ammonium chloride solution = 10.22  $\gamma$  of N.

Ammonia N found	Recovery
$\gamma$	<i>per cent</i>
7.56	74
7.55	74
7.62	74
7.67	75
7.90	77
7.53	74

simplicity and speed of the latter method justify its use for all but the most critical determinations.

*Interference of Ammonia*—It is well known that ammonia is titrated in the formol titration and, if present along with the amino acid, will appear as an error in the determination. The interference from this material may be eliminated by its complete removal (aeration from the basic solution), or more commonly by determining the ammonia separately and subtracting an appropriate correction. Since the ammonia may not be determined quantitatively by the formol titration, the determination of this correction factor must be made by determining the percentage recovery of ammonia under the conditions used. Ammonia has a dissociation constant very close to that of phenolphthalein,



a fact which makes the recovery quite dependent on the shade of color chosen in the adjustment of the first end-point. For this reason, different analysts will obtain different recovery figures which will, however, usually be quite consistent with the particular analyst. Table III shows typical data obtained by one analyst who was running the determination for the first time. It is seen that a reasonable precision is obtained, yielding a negligible variation in the amino acid figures when the ammonia recovery is used merely as a correction.

#### SUMMARY

A drop scale formol titration for determination of amino nitrogen with simple indicators is described.

The results of the indicator method are compared with results obtained by the method of Borsook and Dubnoff in which electrometric titration is used.

The method is shown to be comparable with the macro formol titration in recovery and accuracy, but applicable to very much smaller quantities of material.

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## QUANTITATIVE DROP ANALYSIS

### XIV. POTENTIOMETRIC DETERMINATION OF CHLORIDE\*

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(Received for publication, January 8, 1941)

In connection with a proposed investigation of ion accumulation in *Paramecium caudatum*, it was necessary to have available a quantitative ultramicro method for the determination of chloride in fluids of biological origin, capable of determining down to 2 or 3  $\gamma$  of chloride, with an error not exceeding 1 per cent. Four ultramicro methods for chloride have been described in the literature. Conway (1) has published the details of a diffusion method in which chloride was quantitatively oxidized under suitable conditions, and the chlorine gas thus formed diffused into 20 per cent potassium iodide solution. When more than 35  $\gamma$  of chloride was present, the triiodide formed was titrated with dilute thiosulfate solution. Below 35  $\gamma$  of chloride, the estimation of the triiodide was carried out colorimetrically. As is well known, the error of colorimetric methods, in general, is of the order of several per cent. The Conway method, therefore, was not satisfactory for our needs.

Schwarz (2), in a general description of potentiometric titrations on an ultramicro scale, included detailed directions for the argentometric titration of chloride in amounts ranging from about 5 to 1.5  $\gamma$  of chloride with a precision of about 0.5 per cent. The Schwarz method of titration in a platinum loop is not adapted to samples of various volumes, and does not readily lend itself to quantitative transfer of liquid samples or ash solution to the titration loop. Schwarz did not investigate the application of his method to biological fluids.

\* Aided by grants from the Rockefeller Foundation, the American Philosophical Society, and the Research Board of the University of California.

Linderstrøm-Lang, Palmer, and Holter (3) modified the Schwarz method and made it adaptable to routine handling of biological materials, but, in so doing, raised the lower limit of applicability and diminished the accuracy of the method to such an extent that it was unsatisfactory for the purpose in hand.

Wigglesworth (4) developed a method for determining the chloride content of the blood of a single mosquito larva. It was based upon the addition of excess silver nitrate, filtration, and back titration with 0.01 N sodium thiocyanate. The error of the Wigglesworth method is relatively large ( $\pm 6$  per cent). The lower limit of application cannot be determined from an inspection of his data, since they are given in terms of arbitrary "units." The analytical data given are too meager to evaluate adequately the worth of the method. The author states that the method is applicable to 0.3  $\lambda^1$  of tissue fluid, "or less if required." Presumably this represents about 1  $\gamma$  of  $\text{Cl}^-$ , if plasma is taken as representative.

The present authors were forced, therefore, to devise a method fulfilling the requirements of their problem as stated. It seemed desirable, if possible, to fit this into the framework of the technique and apparatus devised by Kirk and his associates (5-7) in their development of systematic "drop analysis."

The method, as finally devised, consisted of electrometric titration, with standard "drop scale" equipment. No reference half-cell was used, but instead of that, the considerably simpler bimetallic electrode system of Clark (8) was employed. The titration was rapid, simple, and accurate, the standard error being 0.5 per cent or less down to 2  $\gamma$  of chloride, 1 per cent at 1  $\gamma$ , and only 2 per cent at 0.5  $\gamma$ . It was directly applicable, without ashing, to biological fluids containing protein.

#### EXPERIMENTAL

##### *Apparatus*

1. A potentiometer graduated in millivolts, such as the Leeds and Northrup Students' type potentiometer.

2. A galvanometer of medium sensitivity, such as the Leeds and Northrup Lamp and Scale galvanometer, drawing a current of about 1 microampere per scale division.

<sup>1</sup> 1  $\lambda$  = 1 microliter = 0.001 ml.

3. Electrodes were constructed by forming a ball of silver about 2 mm. in diameter on the end of No. 20 silver wire. The electrodes were constructed in pairs, and, before being used, one electrode was immersed in dilute nitric acid until gas escaped freely, after which it was washed in distilled water. The other electrode was repeatedly dipped into a pool of mercury beneath a layer of dilute nitric acid until it was well amalgamated. It was not used for at least an hour after amalgamation. The electrodes thus prepared could be used for several days before recleaning and reamalgamation were necessary. A section diagram of the electrode assembly is given in Fig. 1.

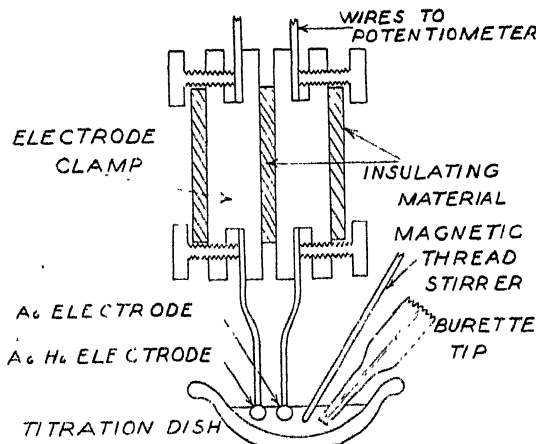


FIG. 1. Titration assembly

4. Burette, pipettes, and titration dishes were described in previous papers of this series (5, 6, 9).

The arrangement of the apparatus for carrying out the titrations is shown in Fig. 1.

*Solutions—*

1. 0.01 N silver nitrate, prepared by dilution from 0.1 N silver nitrate previously standardized against sodium chloride solution.
2. 0.005 N silver nitrate, prepared as above.
3. 0.01 N sodium chloride, prepared by dilution from standard 0.1 N sodium chloride.
4. 1 N sulfuric acid, from redistilled sulfuric acid, free of  $\text{Cl}^-$ .

5. 0.01 *N* sodium bicarbonate, prepared from reagent grade sodium bicarbonate, free of  $\text{Cl}^-$ .

In the preparation of the reagents only the purest water, redistilled from an alkaline solution in an all-Pyrex still, was used.

#### *Procedure*

The sample, containing from 30 to 0.5  $\gamma$  of chloride, was measured by a capillary pipette<sup>2</sup> into a porcelain titration dish (5). Sufficient 1 *N* sulfuric acid was added to bring the final concentration of acid to about 0.3 to 0.4 *N*, as recommended by Zintl and Betz (10). The titration dish was put in place on the titration table, the thread stirrer inserted, and the electrode pairs lowered until they dipped into the chloride solution. It was found that complete immersion was not necessary, and that entirely reliable results could be obtained with the electrodes barely touching the surface of the titration solution. Equal increments of silver nitrate solution were added from the burette, and the potential determined after each addition. As noted by Clark (8), the first small addition of silver produced a marked change in potential, after which the potential remained almost constant, changing only 1 or 2 millivolts per microliter of 0.01 *N* silver nitrate until the titration was about 95 per cent completed. Thereafter, the rate of change of potential increased rapidly until it reached a value of from 25 to 50 millivolts per microliter in the immediate neighborhood ( $\pm 0.2 \lambda$ ) of the end-point. Typical curves are shown in Fig. 2. Equilibrium was not attained immediately, especially near the end-point, but neither the shape of the curves nor the indicated position of the end-point was significantly altered if readings were made after only 30 second equilibration periods, provided that the addition of the increments and the readings of potential were carried out in a regular manner.

In the titration of samples of entirely unknown chloride content, the practice was to add fairly large increments until the approximate position of the end-point was determined. A duplicate titration, in which small increments were then added in the neighborhood of the indicated end-point, served to locate the

<sup>2</sup> The common method of adjusting the meniscus by touching the tip of the pipette to the finger could not, of course, be used for chloride solutions. The meniscus was adjusted by bringing the tip of the pipette in perpendicular contact with a smooth glass plate.

position of this point precisely. The actual potential values found during a titration were not precisely reproducible, even with the same electrode pair in duplicate titrations. With different electrode pairs the E.M.F. values were, of course, markedly different,

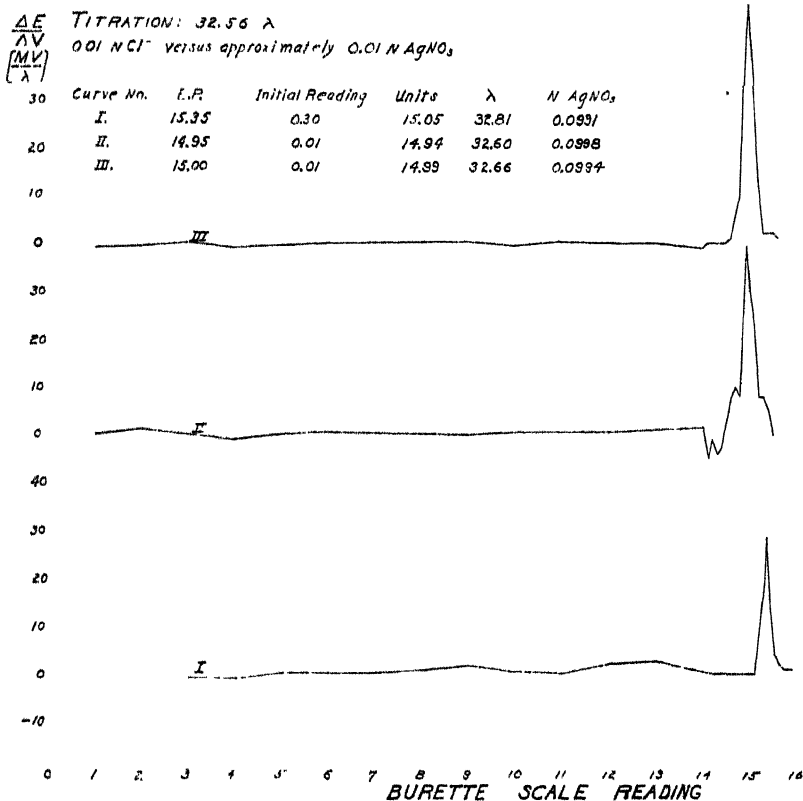


FIG. 2. Titration curves,  $\Delta E/\Delta V$  versus burette reading. E.P. = reading of the burette at the end-point; initial reading = initial reading of the burette; units = E.P. minus initial reading;  $\lambda$  = units times burette scale factor to convert scale reading to microliters.

but it was invariably found that the greatest rate of change in E.M.F. occurred at the end-point. In determination of the actual position of the end-point, the classical method of plotting  $\Delta E/\Delta V$  against  $V$  (where  $E$  is the observed E.M.F. and  $V$  is the volume of standard silver solution added) was used.

In the application of the method to the determination of the chloride content of plasma, two methods were used. First, the titration of the plasma (previously diluted 1:10 to lower the chloride concentration sufficiently) was carried out precisely as described above. In the second method, to the sample of diluted plasma was added an equal volume of 0.01 N sodium bicarbonate solution. The plasma-bicarbonate mixture was evaporated to dryness in a small platinum crucible, ashed at about 500° until white (usually this required from 5 to 10 minutes), cooled, taken up in about 100  $\lambda$  of redistilled water, and about 40  $\lambda$  of 1 N sulfuric acid added. The titration was carried out in the platinum dish in the manner already described. Failure to add the bicarbonate solution to the serum resulted in serious losses of chloride during ashing. Since the ashed samples showed no significant differences in chloride content, as compared to those directly titrated, it was concluded that the plasma proteins did not interfere with the titrations under the conditions described.

### *Results*

In order to determine the precision and accuracy of the method as applied to the determination of various quantities of chloride, a standard 0.1 N solution of sodium chloride was prepared by weighing out 2.9225 gm. of pure (Merck, reagent grade) dry sodium chloride, dissolving this in redistilled water, and diluting to 500 cc. This solution was used to standardize an approximately 0.1 N solution of silver nitrate. The method used in the standardization was to pipette equal quantities of the two solutions into an Erlenmeyer flask and to finish the titration by the dropwise addition of 0.01 N silver nitrate from a calibrated 1 cc. pipette until the further addition of silver nitrate no longer produced a clouding of the solution. The solution was carefully boiled between additions of  $\text{Ag}^+$  in order to produce a perfectly clear solution. As is known, this method is one of great precision when the two solutions are initially of nearly the same normality. From these solutions, working solutions of  $\text{Ag}^+$  and  $\text{Cl}^-$ , in normalities of approximately 0.01, 0.001, 0.005, and 0.0025, were prepared by dilution, carefully calibrated pipettes and volumetric flasks being used.

These solutions were then used to determine the applicability

TABLE I  
*Sodium Chloride Solutions*

Sample		Cl <sup>-</sup> (calculated)	Cl <sup>-</sup> (found)	Standard error		Mean recovery
M	$\lambda$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
0.00999	58.61	20.70	20.67	$\pm 0.03$	$\pm 0.15$	99.9
			20.69			
			20.60			
			20.68			
			20.72			
			20.79			
0.00999	32.56	11.51	11.56	$\pm 0.02$	$\pm 0.17$	100.0
			11.50			
			11.55			
			11.50			
			11.46			
0.00999	22.08	7.81	7.71	$\pm 0.01$	$\pm 0.13$	99.9
			7.80			
			7.86			
			7.82			
			7.82			
0.00499	22.08	3.90	3.89	$\pm 0.01$	$\pm 0.26$	99.7
			3.93			
			3.89			
			3.86			
			3.87			
			3.94			
			3.93			
			1.95			
0.00250	22.08	1.95	1.92	$\pm 0.01$	$\pm 0.51$	99.4
			1.92			
			1.92			
			1.95			
			1.93			
0.00125	22.08	0.97	0.97	$\pm 0.01$	$\pm 1.02$	98.3
			0.97			
			0.96			
			0.93			
			0.93			
0.000625	22.08	0.48	0.47	$\pm 0.01$	$\pm 2.08$	102.0
			0.49			
			0.51			
			0.52			
			0.48			

of the method to the determination of chloride. The results are given in Table I.



In the application of the method to the determination of chloride in human plasma, 10 cc. of whole blood were withdrawn by syringe from an arm vein, sufficient oxalic acid was added to prevent clotting, the blood was centrifuged to remove corpuscles, and an aliquot of the plasma removed for analysis. Before the plasma

TABLE II  
*Plasma Solutions*

Plasma	Sample size	Cl <sup>-</sup> found	Standard error		Cl per 100 cc. plasma	
	$\lambda$	$\gamma$	$\gamma$	per cent	mg.	
Unashed	28.86	10.42				
		10.46				
		10.50				
		10.50				
		10.50				
	22.08	10.51	$\pm 0.03$	$\pm 0.29$	365	
8.05						
8.09						
8.11						
8.10						
Ashed	22.08	8.15	$\pm 0.02$	$\pm 0.25$	366	
		10.58				
		10.52				
		10.46	$\pm 0.03$	$\pm 0.34$	365	
	Cl present	Cl added	Added Cl recovered	Standard error		Mean recovery
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
Unashed	7.81	3.90	3.68			
			3.93			
			3.69			
			4.02			
			4.04			
	10.52	0.63	4.04	$\pm 0.03$	$\pm 0.77$	100.0
			0.65			
			0.58			
			0.61	$\pm 0.04$	$\pm 1.6$	97.0

was analyzed, it was diluted 1:10 in order to make the chloride concentration approximately 0.01 N. In the first series of experiments the plasma was titrated directly in the presence of about 0.4 N sulfuric acid. In the second, the plasma was ashed, as already described, and the chloride determined on the ash. Since the two results were the same within the limit of experimental

error, it was decided that the direct titration gave valid results. In order to establish this point further, samples of plasma, to which known amounts of chloride had been added, were likewise analyzed, and the results checked for recovery of added chloride. The results of these analyses of plasma are given in Table II.

From an inspection of the results it is apparent that the error of the method does not exceed 0.5 per cent if more than 2  $\gamma$  of chloride is present, and that it does not exceed 2 per cent if more than 0.5  $\gamma$  is present. It is equally clear that, in the presence of 0.3 N sulfuric acid, plasma proteins do not affect the titration, since ashed and unashed samples give the same titration values. This is further confirmed by the quantitative recovery of chloride added to plasma. The somewhat greater variation in results in the recovery of added chloride is a reflection of the greater number of operations involved.

#### SUMMARY

1. A method, with previously described equipment, is given for the estimation of chloride in quantities ranging from 20 to 0.5  $\gamma$ . The error does not exceed 0.5 per cent if more than 2  $\gamma$  of chloride is present, and does not exceed 2 per cent if above 0.5  $\gamma$  of chloride is present.

2. The method is applicable, without ashing, to fluids containing protein.

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## QUANTITATIVE DROP ANALYSIS

### XV. DETERMINATION OF POTASSIUM\*

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(Received for publication, January 8, 1941)

In order to carry out the investigation of ion accumulation mentioned in Paper XIV of this series (1), a quantitative ultramicromethod for the determination of potassium was needed. A survey of the literature revealed that no such ultramicromethod has been described, although Linderstrøm-Lang (2) has published the details of a method for estimating sodium and potassium together in quantities of approximately 0.2 to 2  $\gamma$ .

In spite of many careful and complete studies of the procedures involved in the various methods for the estimation of potassium on a micro or macro scale, the error of the estimation in mixtures approximating the composition of the inorganic moiety of biological materials has remained at least 1 per cent, and frequently is greater. The single exception to this statement is the method described by Bullock and Kirk (3).

In those procedures based on the separation of potassium as the cobaltinitrite, it would appear that the error is due to variation in the composition of the precipitate when the conditions of precipitation are altered (4, 5). The precipitation of potassium from a solution of unknown composition would then involve an uncertainty as to the exact composition of the precipitate.

The precipitation of potassium as the chloroplatinate is less capricious in so far as the character of the precipitate is concerned, and the results obtained by Bullock and Kirk on sea water offered a reasonable hope for attempting the determination of potassium

\* Aided by grants from the Rockefeller Foundation, the American Philosophical Society, and the Research Board of the University of California.

in biological fluids on an ultramicro scale. The attempt to apply the Bullock and Kirk procedure directly to quantities of a few micrograms was not successful. If the potassium was evaporated to dryness with an excess of chloroplatinic acid, results were invariably high and very irregular. The precipitation difficulty was solved by dissolving the potassium (as chloride) in 80 per cent alcohol and precipitating it by adding excess chloroplatinic acid. The resultant potassium chloroplatinate was filtered off, reduced with sodium formate, and the  $\text{Cl}^-$  titrated electrometrically by the method of Cunningham, Kirk, and Brooks (1).

It was evident that if the method was to be considered entirely satisfactory, it would have to be applicable to solutions representing the approximate composition of biological fluids; *i.e.*, fluids containing from 10 to 15 times as much sodium as potassium. Accordingly, solutions of known potassium content were made up containing 20 times as much sodium as potassium, and the procedure as outlined was applied to these. The results proved to be quite satisfactory. In order to check the method further, determinations of potassium in serum were carried out.

#### EXPERIMENTAL

##### *Solutions—*

(a) 4 per cent chloroplatinic acid in 80 per cent alcohol was prepared by dissolving the pure acid in 80 volumes per cent of ethyl alcohol.<sup>1</sup>

(b) 80 per cent ethyl alcohol saturated with potassium chloroplatinate.<sup>1</sup> An excess of the finely divided salt was added to the alcoholic solution, with which it was allowed to stay in contact for 12 hours with occasional shaking. At the end of that time, the saturated solution was centrifuged at 1200 to 1500 r.p.m. for 30 minutes, and two-thirds of the clear supernatant solution carefully removed for use. The remainder of the solution was agitated to resuspend the excess potassium chloroplatinate. More 80 per cent alcohol was added and the solution set aside for the preparation of more wash solution.

(c) 95 per cent ethyl alcohol, saturated with potassium chloroplatinate, was prepared by saturating 95 per cent ethyl alcohol

<sup>1</sup> Alcoholic solutions containing chloroplatinate are not indefinitely stable. It is better to prepare not more than a week's supply at a time.

with the potassium chloroplatinate in the manner described above.<sup>1</sup>

(d) 0.2 M sodium formate, prepared by dissolving the pure salt in redistilled water.

(e) 80 volumes per cent of ethyl alcohol.

(f) Standard 0.01 N silver nitrate.

(g) 1 N sulfuric acid in redistilled water.

*Apparatus*---

(a) Standard drop scale equipment, as described in previous papers of this series (6, 7).

(b) Electrometric titration assembly, as described in Paper XIV (1).

*Procedure*

*Inorganic Solutions*—The sample of mixed chlorides was measured by means of a capillary pipette into a small test-tube (20 × 8 mm.) and evaporated by heating in the boiling water bath shown in Fig. 1. The bath consisted simply of a 100 cc. beaker fitted with a metal cover drilled to receive the test-tubes. The walls of the test-tubes were thus entirely surrounded by steam, and evaporation proceeded rapidly. When the solution had been evaporated to dryness, the tubes were removed from the water bath and allowed to cool. The chlorides of sodium, potassium, calcium, and magnesium are all moderately soluble to quite soluble in 80 per cent alcohol, so that samples in the normal range of from 5 to 0.5  $\gamma$  of potassium required but a few microliters of alcohol to dissolve them. It made no appreciable difference if a moderate (up to 2-fold) excess of the solvent was present. During the few minutes required to get the chlorides into solution, the test-tubes were kept stoppered to prevent evaporation of the solvent.

The potassium was precipitated from the dissolved salts by adding a volume of 4 per cent chloroplatinic acid in 80 per cent alcohol equal to the volume used to dissolve the chlorides.

The tubes were stoppered and rotated to bring about an intimate mixing of solution and precipitating reagent. 12 hours at room temperature were allowed to complete the precipitation.

At the end of this time, the excess precipitating reagent was filtered from the potassium chloroplatinate which was washed

twice with 50  $\lambda^2$  of 80 per cent alcohol saturated with potassium chloroplatinate and then with 95 per cent alcohol saturated with the same salt, in order to insure complete removal of excess chloroplatinic acid. The filtration technique deserves special attention, since it proved originally to be a cause of some trouble. Filter sticks made of capillary tubing of 2 mm. internal diameter and 6 mm. external diameter were used. The lower end of the filter stick was tapered and provided with a fused-in 4 mm. section of sintered glass at the tip. In preparing the stick for a filtration, a 1 mm. thick layer of fine asbestos was sucked on to the disk. The asbestos was then pressed on to the disk with the finger nail. A second layer of fine asbestos was deposited on the top of the first by reinserting the filter stick into the asbestos suspension

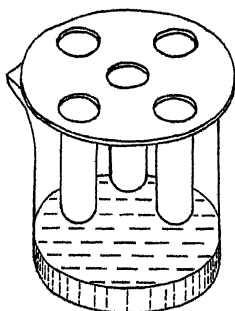


FIG. 1. Steam bath for evaporation of mixed chlorides

(asbestos free of reducing agents, prepared by boiling the material in acid ceric sulfate, was used). A cone of asbestos about 2 mm. in thickness was allowed to deposit.

The technique of using this prepared filter is illustrated in Fig. 2. Only the tip of the cone was allowed to come in contact with the solution. Thus the potassium chloroplatinate collected only on this part of the asbestos. Quantitative transfer of the potassium chloroplatinate could be accomplished simply by disengaging the pad at its base.

The precipitated potassium chloroplatinate was washed twice with approximately 50  $\lambda$  portions of the wash solution. This was run into the test-tube and the tube rotated to bring it in

<sup>2</sup> 1  $\lambda$  = 1 microliter = 0.001 ml.

contact with all the precipitate, part of which was deposited on the walls of the tube.

When the washing was completed, the filter pad was removed and placed in a porcelain titration dish. A few drops of water were added to dissolve the precipitate remaining in the test-tube. The process of solution was hastened by warming the tube. The solution was transferred to the titration dish by means of a capillary pipette, followed by two rinsings with a few drops of warm water. A drop of 0.2 M sodium formate was added, and the solution evaporated to dryness on a steam bath to bring about reduction of the chloroplatinate  $[\text{PtCl}_6^{4-} + 2\text{HCOO}^- + 2\text{OH}^- \rightarrow \text{Pt} + 6\text{Cl}^- + 2\text{CO}_2 + 2\text{H}_2\text{O}]$ . The dish was removed from the

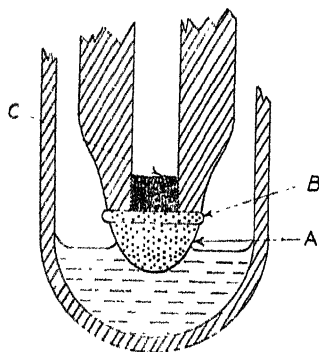


FIG. 2. Filtration detail. *A* represents an asbestos filtering cone; *B* an asbestos base pad; *C* a sintered glass plug.

bath and allowed to cool. A few drops of water were added to dissolve the chlorides and the solution acidified with 2 drops of 1 N  $\text{H}_2\text{SO}_4$ . The titration was carried out as described in the paper on the determination of chloride (1). While formate will reduce  $\text{Ag}^+$ , the rate of the reaction at room temperature is so slow as not to interfere with the titration.

A blank determination in which the entire procedure was carried through as outlined (distilled water being substituted for the sample) had to be made. In the experiments described in this paper the blank was uniformly found to be equivalent to 0.05  $\gamma$  of potassium. The blank value originated from the potassium chloroplatinate of the wash solutions which was not entirely



removed from the walls of the tube, and from the sodium formate which contained traces of  $\text{Cl}^-$ .

The determination of a blank value in procedures involving the electrometric titration of chloride, as described previously (1), has some features which require special mention. The titration of very dilute chloride solution fails to produce a sharp change in potential at the point at which equivalent quantities of the 2 ions are present. Consequently, in a blank titration in which

TABLE I  
*Potassium Solutions*

Sample (22.08 $\lambda$ )	K (calculated)	K (found)	Standard error*		Mean recovery
	$\gamma$	$\gamma$	$\gamma$	per cent	per cent
0.00500	4.32	4.32	$\pm 0.013$	$\pm 0.29$	99.8
		4.30			
		4.27			
		4.33			
		4.33			
0.00333	2.88	2.86	$\pm 0.011$	$\pm 0.38$	99.7
		2.86			
		2.87			
		2.89			
		2.84			
0.000833	0.72	0.71	$\pm 0.012$	$\pm 1.7$	97
		0.71			
		0.71			
		0.69			
		0.68			

\* Standard error =  $\pm \sqrt{\sum d^2 / (n(n-1))}$  where  $d$  = deviation from the mean, and  $n$  is the number of determinations.

very little  $\text{Cl}^-$  is present, smaller quantities of water and acid are added prior to the titration than in the case of a regular determination.

In Table I are given the results of analyses of solutions of known potassium content containing 20 times as much sodium (by weight) as potassium.

*Biological Materials*—The analyses on human blood serum were conducted as follows: The serum was measured by means of a capillary pipette into a small platinum crucible. The organic

matter was destroyed by heating in a micro muffle furnace at about 400°. The ash was dissolved in 0.1 N hydrochloric acid and transferred by pipette to a small test-tube of the type described in the earlier part of this paper. The material was subsequently handled in the manner described for the mixtures of pure sodium and potassium chlorides. The results are given in Table II.

The method described for the estimation of potassium is well suited to routine determinations, the digestions, precipitations, and reductions being carried out in multiple. The filtrations had to be conducted singly, but as these required but a few minutes each they did not greatly increase the average time per determination. Fifteen to twenty analyses could be run in a day.

TABLE II  
*Determination of Potassium in Serum*

Sample $\lambda$	K (found)		Standard error	
	$\gamma$	mg. per cent	$\gamma$	per cent
38.06	7.89	20.6	$\pm 0.03$	$\pm 0.4$
	7.95	20.8		
	7.80	20.4		
	7.85	20.6		
	7.88	20.6		

#### SUMMARY

1. A method is described for the estimation of potassium, suited to the analysis of biological fluids when the ratio of sodium to potassium does not exceed 20.

2. Above 2  $\gamma$  of potassium, the error does not exceed 0.5 per cent, and above 0.7  $\gamma$ , it does not exceed 3 per cent.

3. The method is suited to routine multiple determinations.

Clerical assistance in the preparation of this material was furnished by the personnel of the Works Projects Administration, Official Project No. 65-1-08-62, Unit A12.

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## IDENTIFICATION OF THE MOUSE ANTIALOPECIA FACTOR

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(Received for publication, January 11, 1941)

Recently a new dietary essential required by the mouse has been described (1). When this substance was absent from the diet, young mice soon ceased to grow and became completely bald over large areas of the body. Preliminary concentration of the curative substance which was present in liver has been described. Norris and Hauschildt (2) simultaneously and independently described a similar syndrome in mice which were fed a highly purified diet. In the present communication the identification of the curative material will be described. A preliminary statement of our results has appeared recently (3).

### EXPERIMENTAL

*Assay Technique*—In the early part of this investigation the assay procedure previously described (1) was followed exactly. In the last stages, especially after the crystallization of the active substance, the following modifications were introduced. First, the administration of yeast extract to the depleted animals (as with Diet Y) was discontinued and a purely synthetic mixture of water-soluble vitamins (as in Diet S) was employed throughout the test period. In addition, some of the latest experiments were done with 5 mg. of *dl*-sodium pantothenate per 100 gm. of ration in place of the 1 mg. level used previously. The quantity of pantothenic acid had a decided influence on the occurrence and course of alopecia and, when sufficiently high levels were fed, the disease frequently did not result even in the absence of the present antialopecia factor. However, since the relationship between pantothenic acid and the antialopecia factor requires more study,

the details of our investigation of this relationship will be communicated subsequently.

The omission of yeast extract had one noteworthy result. The restoration of hair did not occur as rapidly in animals on a purely synthetic diet as in those fed the yeast extract ration. Yeast extract could not be included from the beginning of the experiments, for, when this was done, no alopecia resulted. For example, two groups of six mice each were fed the purified ration plus 2 per cent of yeast extract from the beginning of the experiment and no cases of alopecia were observed. This fact suggested that the yeast extract was not devoid of the antialopecia factor.

*Preliminary Concentration*—A statement of the steps employed in bringing about concentration of the active substance will be made in order to illustrate the course of the reasoning which led to the use of phytin. Actual details of the isolation of the active principle will be described below after the effect of phytin has been noted. The source of the vitamin in every case has been the fraction of aqueous liver extract which was insoluble in 70 per cent alcohol; this was the same fraction as previously used. It was designated Fraction A.<sup>1</sup> This material was dissolved in water, dialyzed, and the non-dialyzable portion treated with norit. The active norit filtrate was made alkaline with barium hydroxide and alcohol was added in order to precipitate the active compound. The precipitate was freed of barium and the active substance was rendered dialyzable by heating it with sodium hydroxide. Concentrates prepared in this manner gave the Scherer test for inositol.

*Effect of Phytin*—The properties of the concentrates suggested that the active substance might possibly be some phosphoric acid ester of inositol. Studies on the distribution of the vitamin in natural products had revealed that cereal grains were relatively rich sources. Thus, for example, 2 per cent of oats was sufficient to bring about slow cure of alopecia. For these reasons it was thought justifiable to test the action of phytin. It was thought at the time that the active substance was a lower ester of inositol but that phytin might possess activity. When 100 mg. of phytin per 100 gm. of ration were fed, hair was restored and resumption of growth occurred.

<sup>1</sup> We wish to thank Dr. David Klein of The Wilson Laboratories for gifts of this material.

*Isolation of Antialoppecia Factor*—While the assays of phytin were in progress, a crystalline material was obtained from our best concentrate by precipitation with lead acetate and ammonia followed by purification with norit and crystallization from alcohol. The crystals melted at 214–216° and contained 39.8 per cent carbon. When fed at a level of 100 mg. per 100 gm. of ration, they caused restoration of hair. Subsequent tests with authentic inositol showed that this material also possessed activity.

Many procedures have been tested for the isolation of inositol from liver Fraction A and the one found most satisfactory will be described. 100 gm. of Fraction A were dissolved in water and dialyzed for 18 hours. The non-dialyzable portion was evaporated to 200 cc. and refluxed with 400 cc. of concentrated HCl for 6 hours. The solution was then concentrated under reduced pressure to a syrup, made alkaline with barium hydroxide, and treated with sufficient alcohol to give a final concentration of 75 per cent. The precipitate was filtered off, washed with alcohol, and decomposed by suspending it in 65 per cent alcohol and passing in carbon dioxide. The filtrate from the barium carbonate was concentrated under reduced pressure to about 200 cc. and treated with saturated lead acetate until no more precipitate formed. The precipitate was removed and the resulting filtrate was treated with 100 cc. of saturated lead acetate. Enough ammonia was added to cause complete precipitation. The precipitate was filtered off and washed and then decomposed with a slight excess of sulfuric acid. Lead sulfate was removed and the resulting filtrate was again made alkaline with barium hydroxide dissolved in methyl alcohol and enough ethanol was added to give a final concentration of alcohols of 70 per cent. After the mixture had stood overnight, the precipitate was filtered off, washed, and then decomposed by suspending it in 70 per cent alcohol and passing in carbon dioxide. The barium carbonate was filtered off; the filtrate was concentrated to a small volume under reduced pressure, acidified with sulfuric acid, and filtered through norit. Alcohol was added to the filtrate until crystallization occurred. The crystals were recrystallized from water by the addition of alcohol. 42 mg. of material were obtained which melted at 218°. Inositol in the same bath melted at 218°.

$C_6H_{12}O_6$ . Calculated, C 40.0, H 6.7; found, C 40.2, H 6.7

10 mg. of the crystals were heated with 20 mg. of sodium acetate and 20 cc. of acetic anhydride. The excess anhydride was removed under reduced pressure, the sodium acetate was removed by extraction with water, and the product was recrystallized twice from dilute pyridine; m.p. 212–213°. Inositol hexaacetate in the same bath melted at 213°.

$C_{18}H_{24}O_{12}$ . Calculated, C 50.0, H 5.5; found, C 49.5, H 5.2

*Effect of Inositol*—Simultaneously with the assay of the inositol isolated from liver, authentic inositol was tested. 100 mg. per

TABLE I

*Some Effects of Inositol and Derivatives on Nutritional Alopecia of the Mouse*

Diet	Supplement	Level	No. of animals	No. of recoveries in 18 days	Average gain in weight during test period
		<i>mg. per 100 gm. ration</i>			<i>gm. per day</i>
Y	None		4	1	0.2
"	Phytin	100	2	2	0.7
S	"	100	1	1*	0.4
"	Crystals from liver	100	1	1	
Y	Inositol	100	4	4	0.7
S	"	100	1	1	0.1
Y	"	10	1	1	0.5
"	Phosphorylated inositol	100	2	2	0.4
S	Inositol purified through acetate	100	2	2	0.5

\* Recovery not apparent until after 4 weeks on phytin.

100 gm. of ration were sufficient to bring about restoration of hair and resumption of growth. The inositol (1 mole) was then phosphorylated by heating with pyridine and phosphorus oxychloride (7 moles) and, when this material was purified and assayed, it was found to be as active as the original inositol. Lower levels of inositol have been tried and on the yeast extract ration cures have been obtained with as little as 10 mg. per 100 gm. of ration (Table I).

Since inositol is isolated from natural sources and since relatively large amounts must be fed, it is difficult to prove that a small

amount of impurity is not the active material. In addition, slight change of activity with recrystallization cannot be detected, since no quantitative procedure for the assay of the antialopecia factor is available. It is only possible to observe qualitatively whether or not a substance is active. Recrystallization of inositol from dilute alcohol did not destroy its potency. Furthermore, careful purification of inositol hexaacetate by recrystallization from pyridine and then from alcohol, when followed by hydrolysis and further recrystallization of the free alcohol, did not destroy the activity. For these reasons it is believed that inositol or its phosphoric acid ester is the antialopecia factor.

Since only a small amount of inositol could be isolated from the liver fraction and since the amount obtained was not sufficient to account for the observed potency of the liver, a recovery experiment was performed. 100 mg. of inositol were added to the non-dialyzable portion of 100 gm. of Fraction A and the procedure described above was repeated. 56 mg. of inositol were obtained. It was thus evident that only a small fraction of the inositol present was isolated by our procedure.

#### DISCUSSION

The experiments related above demonstrate that the growth of hair of mice is markedly influenced by inositol or its esters. They further demonstrate that a combined form of inositol occurs in liver. Combined inositol, especially in heart muscle, has been postulated by Winter (4) and by Rosenberger (5) based on amounts isolated before and after autolysis or treatment with alkali. The present work demonstrates that an alcohol-insoluble, water-soluble, non-dialyzable substance occurs in liver which yields inositol upon acid or alkaline hydrolysis. That no free inositol was present in the non-dialyzable concentrates was shown by failure to isolate crystals when acid or alkali treatment was omitted from the procedure.

The relationship of inositol to the growth of hair in the mouse suggests the use of this substance in other species which manifest deficiencies involving the hair. Whether or not inositol is the additional anti-gray hair factor postulated by Dimick and Lepp (6) and by Williams (7) has not been determined.



## SUMMARY

The alopecia which developed in young mice raised on a highly purified diet was cured by addition of inositol or of phytin to the ration. Inositol has been isolated and identified in liver concentrates which cure the same type of alopecia.

Observations on combined inositol in liver have been made.

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# EFFECT OF PREGNANCY ON THE PHOSPHORUS TURN-OVER OF THE SKELETON OF RATS MAINTAINED ON NORMAL AND RACHITOGENIC DIETS\*

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(Received for publication, January 9, 1941)

Dietary phosphorus has been shown to enter rapidly into all calcified tissues of the rat. Within 4 hours after radioactive phosphate administration, significant quantities of the element have been found not only in the epiphyses and diaphyses of the long bones, but even in the molars and tips of the incisors (1). Since the phosphate was undoubtedly blood-borne, these findings show first, that these tissues are permeable, and second, that some of the phosphorus of mineral deposits is readily exchangeable. Some sort of equilibrium would be expected between the marked phosphorus in the blood and the phosphorus in those parts of the tissues undergoing exchange. When equilibrium is reached in certain parts of the adult skeleton, the ratio of marked to total phosphorus in these parts has been shown to be the ratio current in the blood (2). New calcification, taking place after a dose of marked phosphorus has been allowed to come to equilibrium in the body, would have a marked to total phosphorus ratio equal to that of the blood. It is difficult to isolate "new" calcification in adult animals; however, the fetal skeleton *in utero* gives a readily available source of newly formed bone. With the fetal bone as a mirror of the phosphate changes in the maternal blood, a rachitogenic diet was compared with a normal diet to determine

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Some of the data in this paper were presented by one of us (M. L. M.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The substance of this paper was presented by one of us (S. R. L.) before the Western New York meeting of the Society for Experimental Biology and Medicine at Syracuse, October, 1939.

whether this nutritional stress would alter the rate of phosphate turnover in the maternal organism.

#### EXPERIMENTAL

Radioactive  $\text{Na}_2\text{HPO}_4$  was prepared by dissolving radioactive red phosphorus in aqua regia and adding  $\text{Na}_2\text{CO}_3$  to pH 6 to 7. The solution was made up to a convenient volume and aliquots were taken so that a 1 cc. volume would have suitable concentrations for administration to rats by stomach tube. Forty female rats were divided among four groups as shown in Table I. From 2 to 7 days after the administration of a single dose of radioactive

TABLE I  
*Data on Experimental Animals*

	Adequate diet*				Rachitogenic diet†	
	Group 1, control		Group 2, pregnant		Group 3, con- trol	Group 4, preg- nant
	5	10	6	10	4	5
No. of animals .....						
Days between dose and mating....			2	7		7
Total counts per min.‡ in dose for each animal $\times 10^{-3}$ .....	52	98	52	98	86.7	86.7
Total P in dose for each animal, mg.....	20	0.1	20	0.1	11.2	11.2

\* Purina Fox Chow. Daily P intake, 120 mg.

† Steenbock and Black Ration 2965 (3).

‡ On our scale-of-four Geiger-Müller counters.

phosphorus, animals in Groups 3 and 4 were put on the Steenbock and Black diet, Ration 2965 (3), and animals in Groups 2 and 4 were mated. The young from these matings were separated from the mother immediately at birth and were sacrificed by decapitation. The organic material was dissolved from the bones and teeth of the young by extraction with a 3 per cent solution of KOH in ethylene glycol for 1.5 hours (4). The bones, freed from organic material, were then washed by boiling in distilled water, dried, and weighed.

At the same time that the young were obtained, the mother and a control were sacrificed; their blood was weighed and ashed

at 500° with addition of  $\text{HNO}_3$  if necessary. The long bones and teeth from the mothers and their controls were dissected out immediately after death, and were glycol-extracted and dried. The epiphyses and diaphyses of the ashed long bones were separated and weighed. All of the ashed samples from the young, mothers, and controls were dissolved in  $\text{HCl}$ , made up to 2 cc. volume, and the radioactivity determined with a Geiger-Müller counter (5). The phosphorus in the doses of  $\text{Na}_2\text{HPO}_4$  solution was determined by the method of Holtz (6). For calculation of the P\*:P ratios, the phosphorus percentage of the bone ash was taken to be 18 per cent, a figure based on previous analyses of rat bone and tooth substance made in this laboratory<sup>1</sup> and by others (7). For the blood content of phosphorus, the figure 45 mg. per cent was used. This figure is also based on analyses previously made in this laboratory (8). The subdivisions of Groups 1 and 2 (Table I), differing in time elapsed between the phosphorus dose and mating, are not considered separately in Fig. 1 because of the similarity of the data.

#### DISCUSSION

The concentration of radioactive phosphorus in the various tissues has been calculated in terms of the percentage of the dose per mg. of phosphorus in the tissue. This is represented by the symbol P\*:P, the ratio<sup>2</sup> of marked to total phosphorus. The average values of the ratio for the calcified tissues of mothers, young, and non-pregnant controls are given in Figs. 1 and 2 for normal and rachitogenic diets, respectively. Obviously, the ratio P\*:P gives no measure of the total amount of radioactive phosphorus present in a tissue. Nevertheless use of the ratio is desirable in metabolic studies, because it permits comparison of the extent to which equilibrium has been attained (2).

There has been considerable argument in the literature concerning withdrawal of inorganic salts from the maternal skeleton

<sup>1</sup> Hodge, H. C., unpublished data.

<sup>2</sup> It should be noted that the ratio P\*:P has the units of percentage per mg. Such calculation is necessary to correct for differences in the activities of the doses administered and in the weights of the rats employed. Nevertheless, the P\*:P is a measure of, and for the tissues of any single rat, is proportional to the ratio between the *weights* of marked and total phosphorus.

during pregnancy. Because of the constancy of fetal mineral composition (9, 10) irrespective of the adequacy of the maternal diet, the fetus has been assumed to remove large amounts of inorganic salts from the maternal skeleton. Caries in pregnancy has popularly been attributed to decalcification of the teeth in response to fetal needs for calcium phosphates.

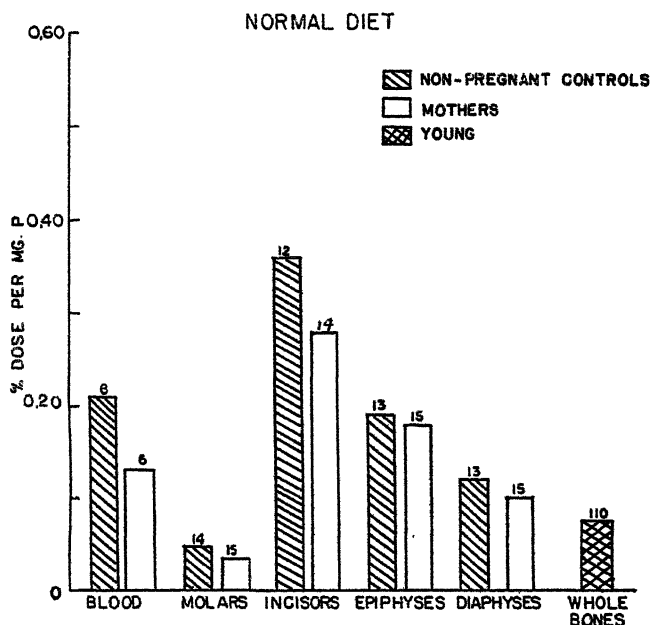


FIG. 1. Normal diet. The percentage of the dose of radioactive phosphorus per mg. of total phosphorus in the blood and calcified tissues of the mothers, young, and non-pregnant controls. The number above each column refers to the number of animals represented by that average.

Since, according to Manly, Hodge, and Manly (2), the labile and stable portions of the hard tissues differ with respect to their  $P^*:P$  ratios, sufficient withdrawal of bone salts from the labile portions should produce a significant change in the  $P^*:P$  ratio for the total bone. It can be seen in Figs. 1 and 2 that there is a tendency for all maternal hard tissues to have lower ratios than those of the controls. These differences, however, are not statistically significant. Nor is there a constant difference be-

tween each experimental rat and the control killed at the same time. There is no evidence in the data presented here that the maternal bones or teeth suffer marked loss of inorganic material during gestation, whether the mother has had an adequate or a deficient diet.

Dietary deficiency *per se*, on the other hand, does produce a marked displacement of the P\*:P ratios as shown by a comparison

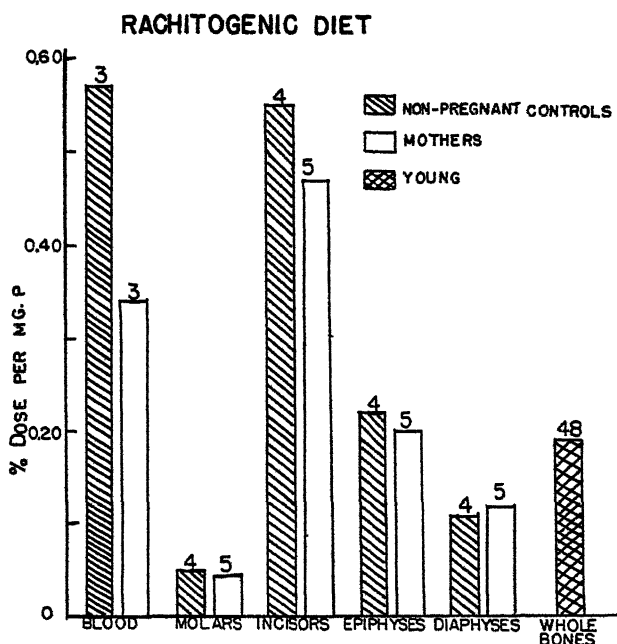


FIG. 2. Rachitogenic diet. The percentage of the dose of radioactive phosphorus per mg. of total phosphorus in the blood and calcified tissues of the mothers, young, and non-pregnant controls. The number above each column refers to the number of animals represented by that average.

of Figs. 1 and 2. It seems probable that the dietary unbalance has not effected a withdrawal of any significant amount of the radioactive element from either the molars or the diaphyses, since their ratios are the same in Figs. 1 and 2. On the contrary, the ratio for the incisors is about 60 per cent higher on the Steenbock diet. Obviously this does not mean the incorporation of more phosphorus in the incisors of rats on an inadequate intake. Since

the incisors are continually calcifying, they must present an integrated picture of the radioactive phosphorus levels in the blood. Hence the higher values for the incisors shown in Fig. 2 indicate that as a whole the  $P^*:P$  ratio in the blood of the deficient animals has been 60 per cent higher than that of the controls. At first this would seem contrary to expectation, since it might be argued that the rickets-producing diet would deplete the phosphorus stores, and hence would diminish the ratios of all tissues having active exchange. However, in the depletion of the phosphorus stores at a time when they are characterized by high  $P^*:P$  ratios, the ratio of the blood is raised, producing high ratios in the maternal incisors and in the actively calcifying bones of the young. The adequate diet which daily furnished 120 mg. of ordinary phosphorus for normal exchange lowers the ratios by washing out the marked phosphorus.

From the indication of the  $P^*:P$  history of the blood given by the incisor values we can deduce evidence of reduced calcification rates in the epiphyses of animals on the Steenbock diet. As pointed out above, the data on the *incisors* of animals on the rachitogenic diet indicate that the  $P^*:P$  ratio of their blood was on the whole 60 per cent higher than that of the controls. The *epiphyseal* ratios, however, are hardly higher in the deficient animals than in the controls, as is seen by comparing the epiphyseal levels in Figs. 1 and 2. A 60 per cent higher epiphyseal ratio would be expected in the deficient animals if similar degrees of calcification took place on both normal and Steenbock diets. Thus, the epiphyseal calcification has been retarded by the deficient diet.

#### SUMMARY

The distribution of radioactive phosphorus has been determined for the calcified tissues of forty female rats which had been previously fed a single dose of radioactive  $Na_2HPO_4$  and subsequently submitted to pregnancy on normal or rachitogenic diets.

1. There is no significant effect of pregnancy, either alone or in combination with dietary deficiency, on the marked phosphorus distribution in the maternal skeleton.

2. The Steenbock and Black diet causes an increase in the  $^{32}P$  fraction of total phosphorus in the actively calcifying tissues.

No change occurs in the marked phosphorus of the molars or diaphyses even though these contain significant amounts of the radioactive isotope.

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## STUDIES ON PITUITARY LACTOGENIC HORMONE

### V. REACTIONS WITH IODINE\*

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(Received for publication, December 27, 1940)

The reactions of proteins with iodine have already interested several investigators. In the instances studied only the tyrosine of the protein reacted with iodine, although an exception was furnished by an instance in which —SH groups were present in the molecule (2). Herriott's (3) work showed that iodine attacks only the tyrosine group of pepsin and that the iodinated pepsin possesses less than 1 per cent of the original enzymatic activity.<sup>1</sup> Similarly, the loss of activity of insulin after being iodinated was observed by Harington and Neuberger (5). They were able to recover the insulin activity after iodine was removed from the tyrosine group by catalytic hydrogenation. These experiments mentioned all indicated that under proper conditions iodine reacts with proteins in a specific manner, as is the case with ketene and cysteine.

Our previous work on the acetylation of the pituitary lactogenic hormone (6) has indicated that the hormone cannot unfold its characteristic biological effects unless the free amino groups are

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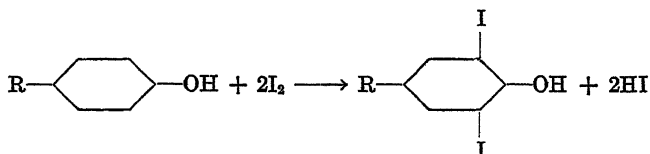
For a brief account of the reactions of iodine with lactogenic hormone see (1).

<sup>1</sup> A recent paper by Philpot and Small (4) indicated that iodine reacts with some unknown groups in pepsin other than the tyrosine. Their results based only on the decrease of Folin's color are not conclusive. Since no confirmations of their observations have yet appeared, their findings are not discussed in this paper.

present. It has been demonstrated that these groups are not important for the physiological action of insulin (7). It seemed to us important to inquire as to whether or not the tyrosine group is essential for the biological activity of lactogenic hormone, as is the case with insulin. The following experiments indicate that iodine has a specific action on lactogenic hormone and that less than 1 per cent of biological activity remained in the iodinated hormone.

The lactogenic hormone preparations used in the present study have already been shown to be single proteins in electrophoresis experiments (8, 9) as well as in solubility studies (10).

*Reactions in Phosphate Buffer*—The iodination process may be conveniently represented by the equation



where R is the other part of the protein molecule. Therefore, an iodinated protein should not react with the Millon reagent. If a protein absorbs iodine and if iodine reacts only with tyrosine, the tyrosine content as determined by the method of Folin and Ciocalteu (11) should be decreased in accordance with the amount of iodine absorbed. Similarly, the nitrogen content of the iodinated protein must be correspondingly lowered.

As is shown in Table I, the total iodine content found in iodinated lactogenic hormone checks well with that calculated from the amount of free tyrosine. In addition, the decrease in nitrogen values corresponds to the increase in iodine. Thus, the calculated iodine content of Preparation L41 is  $(5.84 - 2.00) \times 253.84 / 181.09 = 5.38$  per cent, while the analytical value is 4.96 per cent. If iodine reacts with groups other than tyrosine in the protein, the analytical value for total iodine should be higher than that calculated from the decrease in tyrosine content. Furthermore, it has been demonstrated by Herriott (3) that practically no oxidation by iodine occurs in the reaction. Hence it may be concluded that the action of iodine on the lactogenic hormone is solely with the tyrosine group. Further evidence of this will be presented in later sections.

That iodination inactivated the hormone (Preparation L36A) in this experiment was shown by the fact that 7.5 times the intramuscular minimal effective dose caused no crop stimulation whatsoever, and 1000 times the local minimal effective dose (micro test) produced only a slight border line reaction.<sup>2</sup>

*Experimental*—100 mg. of Preparation L283B<sup>3</sup> were dissolved in 2 cc. of 0.01 N NaOH and the clear solution was diluted to 7.6 cc. by the addition of 0.5 M phosphate buffer<sup>4</sup> of pH 7.0. To this solution 2.4 cc. of 0.1 N iodine solution (with 0.5 M KI) were added.<sup>5</sup> After the mixture stood at room temperature for 1 hour, the excess iodine was removed by a few drops of 0.2 N thiosulfate reagent.

TABLE I

*Reactions of Iodine with Lactogenic Hormone in Phosphate Buffer, pH 7.0, at 20–21°*

Preparation	N	I <sub>2</sub>	Tyrosine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
L283B	15.26	0	5.84
Same, iodinated for $\frac{1}{2}$ hr. (L41)	14.50*	4.96*	2.00
	14.52†	5.38†	
“ “ “ 1 “ (L36A)	14.00*	7.90*	0.00
	14.12†	8.18†	

\* Found.

† Theoretical.

The colorless suspension was then dialyzed against distilled water which was stirred constantly and changed twice daily. The

<sup>2</sup> In the preliminary paper (1) the last figure in the sixth column of Table I should read >1.5 instead of <1.5.

<sup>3</sup> Throughout this paper B is used to designate the hormone derived from beef pituitaries, while S is used for that obtained from sheep. As is shown in previous publications (10, 12), the beef hormone has more tyrosine than that prepared from sheep, and the sheep hormone is more soluble than the beef in acid solution.

<sup>4</sup> It is of interest to note that the lactogenic hormone does not dissolve completely in phosphate buffer, pH 7.0, but a clear solution results if the hormone is dissolved in dilute alkali first and then combined with phosphate buffer of pH 6.5.

<sup>5</sup> The solution became turbid as soon as iodine was added. The pH of the solution remained the same. Probably iodine is a good precipitating agent for lactogenic hormone.

dialysis was carried out in the cold room (5°) and usually required about 4 days. The completeness of the dialysis was judged by the precipitation<sup>6</sup> of the hormone within the dialysis bag. Nitrogen, tyrosine, and iodine contents of the iodinated hormone were then determined.

The semimicro-Kjeldahl method was used for nitrogen determination. The selenium oxychloride-sulfuric acid mixture was the reagent used for digestion.

The total iodine as determined by the methods of Stimmel and McCullagh (13) and of Herriott (3) was essentially the same. Herriott's technique while more convenient requires more material.

Lugg's modification (14) of the Folin-Ciocalteu method was used to determine the tyrosine content. 30 mg. of the protein were dissolved with 0.5 cc. of 5 M NaOH in a small Pyrex test-tube. The sealed test-tube was then put into a steam bath for about 35 hours. The hydrolysate was then diluted with water to 10 cc. in a volumetric flask. 1 cc. of 5 N H<sub>2</sub>SO<sub>4</sub> was added to 4 cc. of centrifuged solution and the determination of tyrosine was carried out essentially as described by Lugg. The color developed was measured in the Cenco-Sanford-Sheard photometer equipped with a blue filter. The tyrosine content of the solution was read off from a calibration curve established by the employment of pure tyrosine.

*Reactions in Acid Solutions*—The rate of iodination of tyrosine depends on the pH of the solvent (3). Below pH 5.0 the reaction was too slow for convenient measurement, which could be done readily at pH 6.0. Above pH 6.0 changes in the pH had very marked influence on iodination rate. Similarly, changes in pH affected the rate of the iodination of lactogenic hormone.

At pH 3.8, dilute iodine did not react with tyrosine but did with tryptophane. In 0.01 N HCl tryptophane still reacted with iodine but at a much slower rate. Lactogenic hormone, however, did not unite with iodine at pH 3.8 nor in 0.01 N HCl, although it contained about 2.5 per cent of tryptophane (12). This is consistent with the recent finding of Anson that tryptophane in chymotrypsinogen does not react with iodine (2).

<sup>6</sup> The extremely low solubility of the hormone in water has already been demonstrated in a previous paper (10).

If a protein contains cysteine, it should unite with iodine in acid solutions (2). Since lactogenic hormone did not take up iodine in 0.01 N HCl solution, it may be concluded that there are no cysteine or —SH groups in the native hormone. The results are summarized in Table II.

Attention may be drawn to the fact that 2 moles of iodine are required to react with 1 mole of tryptophane to form, probably,

TABLE II

*Reactions of Iodine with Tyrosine, Tryptophane, and Lactogenic Hormone in Acid Solutions at 20–21°*

Preparation	Solvent	Initial iodine	Tyrosine	Tryptophane	Lactogenic hormone	Volume	Time of reaction	Iodine lost
		<i>mM per l.</i>	<i>mM per l.</i>	<i>mM per l.</i>	<i>per cent</i>	<i>cc.</i>	<i>hrs.</i>	<i>mM per l.</i>
L16A	0.01 N HCl	9.35	3.32	0.00	0.00	25.0	5	0
L16B	" "	9.35	0.00	2.97	0.00	25.0	17	0
L17A	" "	9.35	0.00	0.00	1.0 (Preparation L293B)	1.0	5	0.90 2.00
L15	0.01 N acetate buffer, pH 3.8	8.41	4.28	0.00	0.00	25.0	2 22	0 0
L18	" "	8.41	0.00	3.98	0.00	25.0	1.5 6½ 118.5	3.40 4.90 7.30
L19	" "	9.35	0.00	0.00	1.0 (Preparation 294B)	1.0	3.5	0

1 mole of diiodotryptophane. No loss in biological potency of the hormone was found after it had been treated with iodine in acid solution; this is consistent with the finding that iodine was not absorbed under these conditions.

*Experimental*—Reactions were carried out at room temperature (20–21°). The disappearance of iodine was followed by titrating 1 cc. of the solution with 0.002 N thiosulfate solution. A typical

example of the iodine reaction with tryptophane may be described as follows: 20 mg. of tryptophane were mixed with 4.21 cc. of 0.1 N iodine solution and diluted to 25 cc. with 0.1 N acetate buffer, pH 3.8, in a volumetric flask. The solution was allowed to stand at room temperature. 1 cc. was pipetted out at certain time intervals and analyzed for free iodine by titration with thiosulfate solution.

*Reactions in Acid Urea Solutions*—Urea has long been considered a denaturing agent capable of destroying the biological activity of a protein (15) or dissociating the protein molecule (16, 17). On the other hand, some proteins behave as native substances in urea solution (18).

Lactogenic hormone, like pepsin (18) does not change its biological activity in 6.66 M urea solution. Preliminary osmotic pressure measurements indicate that its molecular weight does not decrease in urea. It is of course entirely possible that the hormone may have become denatured in urea and yet have retained its biological potency.

The denatured form of a protein may sometimes show the presence of —SH groups not detectable in the native protein (19). 1 per cent lactogenic hormone, which stood in 6.66 M urea containing 0.01 N HCl, did not absorb iodine. Even though the urea might have denatured the hormone, no —SH groups were detectable. It would appear, therefore, that —SH groups are absent from the lactogenic hormone.

*Experimental*—10 mg. of Preparation L298S were dissolved in 0.8 cc. of 0.01 N HCl-urea (6.66 M) solution. After the solution had stood at room temperature for 24 hours, 0.2 cc. of 0.1 N iodine was added. The whole mixture was allowed to stand at room temperature again for 5 hours. The solution was then titrated with 0.002 N thiosulfate solution. Two control experiments were carried out side by side: in one no lactogenic hormone was used and in the other no iodine. Results show that no iodine reacted with the hormone or with urea. A 0.1 N iodine solution in 6.66 M urea stood at room temperature for a month without any indication that the urea reacted with the iodine.

*Reactions in Urea Phosphate Buffer*—In about 45 minutes, iodine was completely used up by tyrosine, when equal moles of iodine and tyrosine were dissolved in 6.66 M urea solution of pH

6.85 with 0.1 M phosphate buffer at room temperature. Similarly, 1 per cent lactogenic hormone<sup>7</sup> in the same solvent takes up  $2.65 \times 10^{-3}$  mole of iodine in about 40 minutes. The solution (after being dialyzed) was analyzed for iodine and free tyrosine. The iodine was found to be 3.4 per cent; the calculated value based on the free tyrosine (3.0 per cent) was 3.6 per cent, which checks well with the value obtained from the iodine originally added to the reaction mixture. It thus appears that iodine acts only on the tyrosine group of the hormone and that the reaction kinetically resembles that involved in the formation of diiodotyrosine.

Fig. 1 shows the rates of iodination of tyrosine and of the hormone at 1.5°. Both substances absorbed iodine initially at exactly

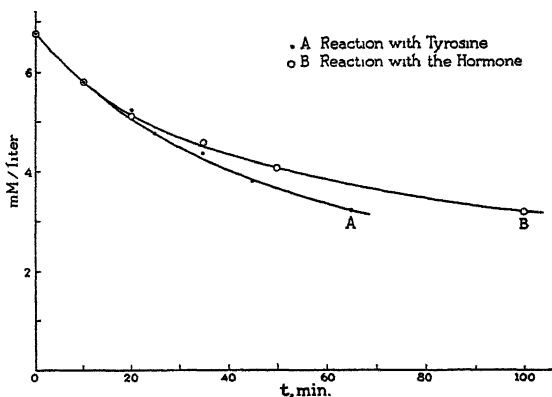


Fig. 1. Reaction rates of iodination of tyrosine and the hormone at 1.5°

the same rate. This was especially interesting, since it is well known that steric hindrance occurs even in simple organic compounds. Possibly certain phenolic groups in the molecule do not associate with other groups to form salt bridges, as suggested by Mirsky and Pauling (20), or they stretch to such an extent on the surface of the molecule that there is no spatial interference from the rest of the molecule. This may also be in accordance with the observations of Holiday (21) who found that on the basis of spec-

<sup>7</sup> Since the beef hormone contains 5.84 per cent tyrosine, 1 per cent hormone solution is equivalent to  $3.2 \times 10^{-3}$  mole of tyrosine. Therefore,  $6.4 \times 10^{-3}$  mole of iodine is required to iodinate completely the tyrosine groups in the hormone.



trographic analysis tyrosine has the same intensity of absorption when bound into the protein chain as it has in the free state. However, after about one-third of the iodine had been used up, iodination of the hormone was slower than that of tyrosine. This suggests that the tyrosine groups in the hormone may occur in at least two different structural configurations, in one of which the phenolic groups can rotate freely. In oxidation and reduction studies, Anson<sup>8</sup> has also observed that all tyrosine groups in a protein do not behave identically.

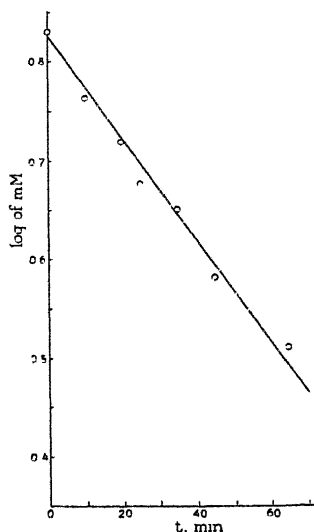


FIG. 2. A plot of time against the concentration of iodine for the iodination of tyrosine at 1.5°.

It is interesting to note that a straight line was obtained from Table V by plotting time against the logarithm of iodine (see Fig. 2). This suggests that the reaction is independent of the iodine concentration

$$-\frac{d(I_2)}{dt} = kC_{\text{tyrosine}}^n C_{\text{iodine}}^0$$

where  $k$  is the specific rate constant. Results from preliminary

<sup>8</sup> Anson, M. L., private communication.

experiments with varied iodine concentrations having a constant tyrosine content confirmed this finding.

*Experimental*—50 mg. of Preparation L293B were dissolved in 5.0 cc. of 6.66 M urea solution, pH 6.85; 0.1 M phosphate buffer and 0.3 cc. of 0.0935 N iodine (in 6.66 M urea) were added. The solution was allowed to stand at room temperature until the iodine disappeared. It was then dialyzed and analyzed for iodine and tyrosine as described above. Similarly, 15 mg. of tyrosine and 1.75 cc. of 0.0935 N iodine were dissolved and diluted with the same buffer to 25 cc. The results are summarized in Table III.

For the reactions at 1.5°, experiments were carried out in the following manner. Hormone solution was prepared by dissolving 214 mg. of Preparation L293B in 10 cc. of the buffer and 10 cc. of 0.027 N iodine solution with the same buffer; they were then

TABLE III

*Reactions of Iodine with Tyrosine and Lactogenic Hormone in Urea-Phosphate Buffer, pH 6.85, at 21–22°*

Preparation	I <sub>2</sub>	Tyrosine	Hormone (Preparation 293B)	Reaction mixture	Time of reaction	I <sub>2</sub> disappearance
	<i>mM per l.</i>	<i>mM per l.</i>	<i>mg.</i>	<i>cc.</i>	<i>min.</i>	<i>mM per l.</i>
L10	3.20	3.32	0.0	25.0	5	1.70
					45	3.20
L13	2.65	0.0	50.0	5.0	40	2.65
L23	2.65	0.0	50.0	5.0	45	2.65

set in the thermostatically controlled water tank at 1.5°. After about 1 hour, the two solutions were mixed in a 25 cc. volumetric flask. 1 cc. of the mixture was pipetted out immediately into 5 cc. of 0.1 N HCl to stop the reaction and titrated with 0.001 N thiosulfate with a 1 per cent starch solution as the indicator. The same titration was carried out at different time intervals. The titrated solutions were then dialyzed and analyzed for iodine and free tyrosine. The results are summarized in Table IV, while Table V shows the results obtained from the reaction of iodine with tyrosine.

*Reactions in Urea Citrate Buffers*—It has already been shown that in urea phosphate buffers of pH 6.85 the iodination of lactogenic hormone resembles that of tyrosine. With a urea citrate buffer of pH 6.50, this finding was confirmed.

51.7 mg. of Preparation L299S<sup>9</sup> were dissolved in 6.66 M urea citrate buffer<sup>10</sup> of pH 6.50. 0.4 cc. of 0.0935 N iodine solution was added and the final volume was 5 cc. 2.0 cc. of the same iodine solution were added to a solution of 15 mg. of tyrosine in the same buffer in a final volume of 25 cc. Both reactions were

TABLE IV

*Reactions of Iodine with Lactogenic Hormone in Urea-Phosphate Buffer, pH 6.85, at 1.5°*

Amount of Preparation L293B in 20 cc. of solvent = 214 mg.; concentration of iodine =  $6.75 \times 10^{-3}$  M.

Time	I <sub>2</sub>	Iodine absorbed in 1 mg. protein		Free tyrosine determination
		Calculated	Found	
min.	mM per l.	$\gamma$	$\gamma$	per cent
0	6.75	0	0	5.6
10	5.78	11.60		4.8
20	5.15	19.0	18.7	
35	4.60	24.44		3.8
50	4.10	31.31	30.5	
100	3.20	39.77	40.0	2.9

TABLE V

*Reactions of Iodine with Tyrosine in Urea-Phosphate Buffer, pH 6.85, at 1.5°*

Tyrosine =  $3.32 \times 10^{-3}$  M; iodine =  $6.75 \times 10^{-3}$  M.

<i>t</i>	I <sub>2</sub>
min.	mM per l.
0	6.75
10	5.80
20	5.25
25	4.75
35	4.40
45	3.80
65	3.25

stopped after 34 minutes. An excess of 0.1 N thiosulfate was added to the hormone solution. The solution was then dialyzed and analyzed for iodine and free tyrosine, while the tyrosine solu-

<sup>9</sup> Sheep hormone contains 4.75 per cent tyrosine (*cf.* (12)).

<sup>10</sup> The buffer was prepared by dissolving 6.1 gm. of citric acid and 200 gm. of urea in 79 cc. of N NaOH and making up to 500 cc.

tion was titrated with 0.002 N thiosulfate. Experiments were carried out at room temperature.

Results showed that  $1.07 \times 10^{-3}$  M of tyrosine was used up in the tyrosine solution. If we assume that an equivalent amount of tyrosine in the hormone was iodinated, the iodinated hormone should have 2.6 per cent of iodine. The iodine found in the hormone was 2.5 per cent, while that calculated from the free tyrosine (3.0 per cent) was 2.6 per cent. Again it appears that iodine reacts with the hormone solely by forming diiodotyrosine.

*Isoelectric Point of Iodinated Hormone*—Isoelectric points of proteins are determined by the dissociation constants of the basic and acid groupings in the molecule. A change of one of these dissociation constants may be expected to shift the isoelectric point in either direction, depending on which group participated.

TABLE VI

*Electrophoretic Mobility of Iodinated Hormone in Different pH Buffers at 1.5°*

Buffer	pH	$\mu \times 10^5$
Citrate.....	3.20	+5.29
".....	3.99	+2.83
Phosphate.....	5.83	-3.92
".....	6.53	-6.05

That the deamination of proteins changes the isoelectric point (22, 23) to the acid side is a typical example.

As is shown in Table VI, the isoelectric point of the completely iodinated lactogenic hormone is about pH 4.7, while the non-iodinated hormone has an isoelectric point at about pH 5.8 (9). The change corresponds to expectation. It may be recalled that the pK of the phenolic group of tyrosine is changed from a value of 10.28 to 6.48 by the introduction of iodine into the benzene, as demonstrated by Dalton *et al.* (24). Furthermore, the work of Neuberger (25) and others has shown that when proteins are iodinated the titration curve of the tyrosine phenolic hydroxyl group is shifted toward the acid region.

*Experimental*—The completely iodinated sheep hormone (Preparation LI20) was prepared as described in the first part of this paper. It contained 6.6 per cent of iodine and had no free tyrosine. A total dose of 5 mg. (at least twenty-five minimal effective

doses) given intramuscularly to squabs 1 month old showed no crop-stimulating activity.

Electrophoresis experiments were carried out in a Tiselius apparatus at 1.5°. Results indicated the presence of a single sharp boundary in all experiments; the whole field was scanned. Mobilities as a function of pH are summarized in Table VI. The isoelectric point was estimated from these data and found to be about pH 4.7.

#### DISCUSSION

Biologically active proteins which do not contain prosthetic groups are not uncommon. Insulin and pepsin are typical examples. The pituitary lactogenic hormone may also belong to this class, as it has not yet been shown<sup>11</sup> to have a prosthetic group. Yet studies of the effect on biological activity of specific modifications of the molecule may enable us to detect "functional" molecular groupings.

That the free amino groups<sup>12</sup> are essential for the biological activity of the lactogenic hormone has already been demonstrated (6, 27). Results herein reported indicate that iodination of the tyrosine group in the hormone markedly decreases the biological activity. Studies of pepsin and insulin show that tyrosine is an essential component of both these substances; yet free amino groups are not essential for their biological action. Lactogenic hormone would seem to be the first protein substance in which both tyrosine and free amino groups have been shown to be essential.

#### SUMMARY

The reaction of iodine with lactogenic hormone has been studied in different solvents at varied pH. The reaction of iodine with tyrosine was also studied in some of these solvents. The results may be summarized as follows:

1. Iodinated lactogenic hormone is almost completely inactivated.
2. The action of iodine on the hormone is only on the tyrosine molecule.

<sup>11</sup> Our unpublished work and that of White and Lavin (26) indicated that the ultraviolet absorption spectrum of lactogenic hormone has the same pattern as for insulin and pepsin.

<sup>12</sup> The free amino groups are probably the  $\epsilon$ -amino groups of lysine.

3. That the tyrosine group is essential for the biological activity of the hormone is thus indicated.

4. Kinetic data suggest that some tyrosine groups of the hormone react with iodine at the same rate as pure tyrosine, whereas other tyrosine groups react more slowly.

5. At pH 3.8 or lower, there is no reaction between iodine and the hormone. It is concluded that no —SH groups are present in aqueous or in 6.66 M urea solutions.

6. The isoelectric point of the iodinated hormone is shifted from pH 5.8 to about pH 4.7 in accordance with expectation.

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## DISTRIBUTION OF NITROGEN AND PROTEIN AMINO ACIDS IN HUMAN AND IN COW'S MILK\*

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The belief that human breast milk is superior to cow's milk in the nutrition of the human infant is held by many pediatricians. The theory is based almost wholly upon clinical experience (1, 2). Studies of the amino acid composition of the proteins of cow's milk have been numerous but little information is available concerning the composition of human milk proteins. The present study of the constitution of these proteins was undertaken, first, because the milk proteins which are elaborated for the young at a time when requirements for growth are at a maximum might be expected to have the ideal pattern of amino acid distribution for supporting rapid growth, and, second, because a comparative study of the composition of cow and human milk proteins might amplify our knowledge of their relative nutritive values. While amino acid composition is not the only factor determining the biological value of proteins, its importance cannot be minimized.

### EXPERIMENTAL

The distribution of the casein, whey protein, and non-protein nitrogen in the total nitrogen of milk samples was determined by an adaptation of the methods of Rowland (3).<sup>1</sup>

\* Preliminary reports of this investigation were presented before the meetings of the American Institute of Nutrition, at Toronto, April 26, 1939, and the Society for Pediatric Research, at Skytop, Pennsylvania, April 26-27, 1939.

<sup>1</sup> Total nitrogen was determined by the macro-Kjeldahl method, on 5 ml. of milk. To determine the non-casein nitrogen 20 ml. of milk were placed in a 100 ml. volumetric flask. 60 ml. of water at 40° were added, followed by 2 ml. of 10 per cent acetic acid. After 10 minutes, 2.0 ml. of



The protein fractions of cow's milk were prepared from three lots of mixed milk obtained from the same herd of cows. The human milk samples were mixtures of milk obtained from mothers producing large volumes of milk, known to be in the mature stage of lactation. No milk was taken earlier than 8 weeks or later than 6 months post partum.

Casein fractions were prepared by the method of Van Slyke and Baker (4).<sup>2</sup> The soluble protein fractions of the milk were prepared from the whey remaining after the precipitation of casein. The whey was placed in collodion sausage casing sacs with thymol as a preservative and dialyzed with running tap water until the test for reducing sugar with Benedict's reagent was negative. About 16 to 18 hours were sufficient to complete the dialysis. (It was found that protein preparations made from whey which was not dialyzed were contaminated with large amounts of carbohydrate.) After dialysis the whey was mixed with a 33 per cent by weight solution of trichloroacetic acid in sufficient amount to bring the resulting mixture to a concentration of 8 per cent trichloroacetic acid. After 30 minutes the whey protein fraction

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normal sodium acetate were added. The mixture was allowed to cool to room temperature, diluted to volume, and filtered. A macro-Kjeldahl determination was made on 50 ml. of the filtrate to ascertain the amount of non-casein nitrogen present. To determine the non-protein nitrogen 10 ml. of milk were placed in a 50 ml. volumetric flask and made to volume with 15 per cent trichloroacetic acid. After 30 minutes the mixture was filtered and the nitrogen contents of 10 ml. aliquots of the filtrate determined by the Van Slyke manometric micromethod.

<sup>2</sup> The milk was centrifuged at high speed until no further separation of fat could be obtained. The fat separated readily from cow's milk but, frequently, difficulty in separating was encountered with freshly drawn human milk, probably owing to the fine dispersion of the fat globules. Upon being held in refrigeration for 24 hours the fat could be removed completely enough to permit a sharp precipitation of casein. 1 liter of fat-free milk was diluted with an equal volume of distilled water and placed in a 3 liter jar. A mixture of 1 part of normal hydrochloric acid and 2 parts of normal acetic acid was introduced very slowly, during efficient mechanical stirring, through a capillary extending below the surface of the milk, until the isoelectric point of casein was reached. The precipitate was washed, and then purified once by dissolving with 0.1 N sodium hydroxide (pH 7), as described by Van Slyke and Baker (4). The casein was washed with distilled water, then with 45 per cent alcohol, finally with 95 per cent alcohol and ethyl ether, and dried in a vacuum oven at 70°.

was centrifuged off and suspended in 95 per cent alcohol. A half volume of ether was added to the alcohol-whey protein suspension and the whey protein centrifuged off, washed with ethyl ether, and transferred to a desiccating vacuum oven at 70° for drying.

TABLE I  
*Chemical Composition of Milk*

The values, measured in gm. per 100 ml., represent averages obtained from the analyses of three different samples of cow's milk and five samples of human milk.

	Cow	Human
Fat.....	3.89	4.12
Total nitrogen.....	0.5176	0.1616
Ash.....	0.780	0.210
Calcium.....	0.1240	0.0318
Magnesium.....	0.0122	0.0038
Potassium.....	0.1550	0.0498
Sodium.....	0.0497	0.0113
Phosphorus.....	0.0944	0.0134
Chlorine.....	0.1050	0.0322
Sulfur.....	0.0326	0.0139

TABLE II  
*Average Nitrogen Distribution in Milk*

	Cow's milk	Human milk
	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>
No. of samples.....	3	4
Total nitrogen.....	517.6	161.6
Casein ".....	404.6	48.6
Whey protein nitrogen.....	88.8	77.0
Non-protein ".....	24.2	36.0

The milk proteins were analyzed for total nitrogen content by the macro-Kjeldahl procedure and for total sulfur as sulfate after combustion in the oxygen bomb. The basic amino acids were determined by the methods of Block (5) with nitranilic acid as the precipitant for histidine. Correction factors were applied to the arginine and lysine values to allow for solubility losses in the micro adaptation of the method. These are described in the foot-

notes to Table III.<sup>3</sup> Tyrosine and tryptophane were determined by the method of Folin and Marenzi (6). Cystine was determined through its cuprous mercaptide by the method of Graff, Maculla,

TABLE III  
*Amino Acid Composition and Nitrogen Content of Milk Protein Fractions\**

	Casein		Whey protein	
	Cow	Human	Cow	Human
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Total N.....	14.50 ± 0.27	14.62 ± 0.14	14.20 ± 0.05	13.73 ± 0.15
Histidine.....	1.81 ± 0.23	1.80 ± 0.06	1.41 ± 0.15	1.13 ± 0.08
Arginine†.....	3.79 ± 0.20	3.31 ± 0.05	3.46 ± 0.08	5.18 ± 0.16
Lysine†.....	6.20 ± 0.06	5.21 ± 0.09	7.91 ± 0.30	5.80 ± 0.16
Tyrosine.....	6.01 ± 0.13	6.11 ± 0.32	4.70 ± 0.11	5.20 ± 0.05
Tryptophane.....	1.11 ± 0.07	1.69 ± 0.14	1.81 ± 0.10	2.32 ± 0.09
Cystine.....	0.26	0.73 ± 0.07	2.49 ± 0.02	3.09 ± 0.31
Methionine.....	3.10	2.19	2.73	1.91
Cystine S.....	0.069	0.195	0.662	0.825
Methionine S.....	0.666	0.471	0.586	0.410
Cystine S + methionine S.....	0.735	0.666	1.248	1.235
Total S.....	0.796	0.682	1.262	1.298

\* Casein of cow's milk represents the average obtained on the analysis of two preparations. The other protein fractions are averages of three preparations. The plus-minus figures represent the approximate range of values among the preparations. No range is given for total sulfur or methionine, since these results were obtained on mixed preparations, owing to insufficient material.

† The arginine values are corrected for loss through solubility of arginine silver by the factor 3.6 mg. per 100 ml. proposed by Gulewitsch (10). For instance, in the present adaptation of the Block method a volume of 325 ml. used (which includes mother liquor and washings) gives a solubility loss of 11.8 mg. of arginine.

‡ The lysine values are corrected for the constant loss of 8.34 mg. of lysine mostly through the solubility of lysine phosphotungstate. This factor was determined by Tristram (11).

and Graff (7) and methionine by the methods of Baernstein (8) with some modifications introduced by Kassell and Brand (9).

<sup>3</sup> It is recognized that small losses of histidine, also, are incurred in the procedure. Work is in progress on the estimation of histidine by the nitranilic acid procedure and special attention is being given the determination of an accurate correction factor.

The fat content of the milk samples and their contents of the mineral elements calcium, magnesium, sodium, potassium, phosphorus, chlorine, and sulfur were also determined.

### Results

The lipid, nitrogen, and mineral ash constituents of cow and human milk samples are shown in Table I. The distribution of total nitrogen in cow and human milks is shown in Table II. Differences in the distribution are striking, human milk containing

TABLE IV  
*Amino Acid Composition of Milk*

	Cow				Human		
	Casein	Whey protein	Total		Casein	Whey protein	Total
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 85.5 ml.*	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Nitrogen.....	404.6	88.8	493.4	125.6	48.6	77.0	125.6
Histidine.....	50.5	8.8	59.3	15.1	6.0	6.3	12.3
Arginine.....	105.7	21.6	127.3	32.4	11.0	29.0	40.0
Lysine.....	173.1	49.5	222.6	56.7	17.3	32.4	49.7
Tyrosine.....	167.9	29.4	197.3	50.2	20.4	29.2	49.6
Tryptophane.....	31.3	11.3	42.6	10.8	5.7	13.0	18.7
Cystine.....	7.3	15.5	22.8	5.8	2.5	17.3	19.8
Methionine.....	86.6	17.1	103.7	26.4	7.2	10.9	18.1
Cystine S.....	1.9	4.1	6.0	1.5	0.7	4.6	5.3
Methionine S.....	18.6	3.7	22.3	5.7	1.5	2.3	3.8
Cystine S + methionine S.....	20.5	7.8	28.3	7.2	2.2	6.9	9.1

\* 25.5 ml. of cow's milk contain as much protein nitrogen as 100 ml. of human milk.

only 30 per cent as much total nitrogen and only 25 per cent as much protein nitrogen as does cow's milk. The mineral content of human milk, shown in Table I, is correspondingly low. In the two milks the whey protein occurs in approximately equal concentrations but the casein content of cow's milk is 8 times as great as that of human milk. 82 per cent of the protein nitrogen of cow's milk was found to be in the form of casein, while in human milk only 39 per cent was contained in the casein fraction, results which are similar to those found in the literature.

The amino acid composition of the casein and whey protein fractions of cow and human milks is shown in Table III. The differences in amino acid composition are not great among the protein fractions of the two milks. The casein fraction of human milk has a significantly higher tryptophane content and is higher in cystine and lower in methionine than cow casein. The whey protein of human milks is significantly higher in arginine and cystine and lower in lysine and methionine than cow whey protein. While these data differ somewhat from those of Plimmer and Lowndes (12) in minor details, the results are similar. The analyses of cow's milk casein for basic amino acids agree with values obtained by Vickery and White (13).

The quantities of each of the seven amino acids and nitrogen in the mixed proteins of 100 ml. of each of the two kinds of milk have been calculated from the data in Tables II and III and are presented in Table IV. It is apparent that all of the amino acids with the exception of cystine occur in much higher amounts in cow's milk. Approximately equal quantities of cystine occur in 100 ml. of each of the two kinds of milk.

#### DISCUSSION

To obtain a comparison of the relative amino acid composition of the mixed proteins of cow and human milks the data must be calculated on the basis of equal amounts of protein nitrogen. For this reason Table IV has a column showing the amino acid composition of 25.5 ml. of cow's milk, which contain a quantity of protein equal to that in 100 ml. of human milk. In spite of the differences between the two milks, in the distribution of protein nitrogen between casein and whey proteins, there is a surprising similarity in contour of the amino acid composition of the protein mixtures, with respect to histidine, arginine, lysine, tyrosine, and tryptophane. It is interesting to speculate whether the amino acid pattern of whole milk protein is ideal for growth purposes and therefore is repeated in the two species. The similarity in composition of the milk protein mixtures of the two species is not carried out with respect to cystine and methionine although the amounts of sulfur furnished by the proteins of the two milks are approximately equivalent.

# SUMMARY

1. Samples of human and cow's milk were examined for their lipid and mineral content and the distribution of total nitrogen in casein, whey protein, and non-protein nitrogen.

2. Samples of casein and whey protein were prepared from cow and human milks and analyzed for seven amino acids: histidine, arginine, lysine, tyrosine, tryptophane, cystine, and methionine.

3. In the proteins of cow's milk the preponderance of sulfur is in the form of methionine, with very little in the form of cystine, while in the proteins of human milk the sulfur is about equally divided between cystine and methionine.

4. The amounts of the seven amino acids contained in the proteins of 100 ml. of human and cow's milk, respectively, were calculated to be 12 and 59 mg. of histidine, 40 and 127 mg. of arginine, 50 and 223 mg. of lysine, 50 and 197 mg. of tyrosine, 19 and 43 mg. of tryptophane, 20 and 23 mg. of cystine, and 18 and 104 mg. of methionine.

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# CEPHALINS, CHOLINE-CONTAINING PHOSPHOLIPIDS, AND TOTAL PHOSPHOLIPIDS IN NORMAL HUMAN PLASMA\*

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Several reports on the concentration of individual phospholipids in normal human plasma have been made in recent years (3, 5, 7-9). Values for sphingomyelins show the greatest differences, probably because of the different criteria used for distinguishing them (insolubility in ether (5), weight of the precipitate with Reinecke acid (8) or of the acetone-insoluble fraction of the precipitate (7), or phosphorus content of the precipitate (3)). But even if only figures obtained by the same authors using the same methods are considered, individual variations are often so great that one might reasonably attribute them to inaccuracies in the extraction and purification of lipids (see also (6)).

Procedures which may reduce or avoid these errors have been investigated. A series of determinations on a fairly large number of individuals was then made, and, in connection with their possible rôle in the transport of fatty acids, postabsorptive values of individual plasma phospholipids have been compared with those obtained after the ingestion of milk fats.

## EXPERIMENTAL

While it is doubtful whether the ether-insoluble fraction of phospholipids represents only sphingomyelins, the determination of the latter by their more specific precipitation with Reinecke acid, as described by Thannhauser and Setz (8), requires large

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samples of blood.<sup>1</sup> Therefore in the present investigations the separate evaluation of sphingomyelins has been omitted, and only the choline-containing (lecithins plus sphingomyelins) and total phospholipids have been directly determined, cephalins being calculated by difference.

Blood was collected from young healthy students in the morning, after fasting, or 3 hours after taking cream ( $\frac{1}{4}$  to  $\frac{1}{2}$  pint) or milk ( $\frac{3}{4}$  to 2 pints). Sodium fluoride or, in a few cases, heparin has been used as anticoagulant. The determinations were made from March through September, 1940.

*Extraction and Purification of Lipids*—In the writer's opinion, the main errors in previous determinations may have resulted from: (a) incomplete or irregular recovery of phospholipids when petroleum ether was used to purify the lipid extracts. Data on this point obtained in this laboratory are omitted because they coincide with others recently published ((3), see also (1)); (b) the presence in the lipid extracts of contaminants which are evaluated as choline, and therefore give too high figures for choline-containing phospholipids. The possible presence of carbon- or phosphorus-containing substances other than phospholipids should also be considered, if total phospholipids are calculated from the carbon of the acetone precipitates or from the phosphorus of the lipid extracts.

With these ideas in mind, the following methods have been compared experimentally: (a) extraction with cold and subsequently with hot alcohol in a continuous extraction apparatus of the Kumagawa-Suto type; (b) precipitation of plasma proteins by 55 per cent saturation with ammonium sulfate at an apparent pH of 3, washing with the acid-salt solution, and subsequent extraction of the precipitate with hot alcohol as above; (c) and (d) precipitation with "colloidal iron" and magnesium sulfate, washing with the saline solution, and extraction of the precipitate with cold alcohol-ether (4).

In methods (a), (b), and (c), the alcohol or alcohol-ether was subsequently evaporated under reduced pressure and the dry residue reextracted with chloroform and filtered through asbestos,

<sup>1</sup> Unlike our previous attempts (1), recent experiments of Erickson *et al.* (3) led to a microprocedure based on the precipitation of sphingomyelins with Reinecke acid. However, the method was published after the completion of the present analyses.

whereas in method (d) the treatment with chloroform was omitted.

### *Procedures*

Phosphorus and choline have been estimated and phospholipids calculated as previously described (1). Total fatty acids plus unsaponifiable substances have been evaluated oxidimetrically (according to Bloor (2)) after saponification, extraction with peroxide-free ether, and purification with dry petroleum ether. Duplicate determinations were always made for choline and fatty acids, and frequently for phosphorus.

### DISCUSSION

Data on the phosphorus and choline contents of lipids extracted and purified by different methods (Table I) show that the extraction of phospholipids is probably complete with all the procedures tested, but washing of the protein-lipid precipitate with the saline aqueous solutions frequently removes substances which would otherwise be computed as choline. On the other hand, with methods (c) and (d), though the same values for choline are obtained, slightly higher figures for phosphorus are occasionally found when the chloroform purification is omitted.<sup>2</sup>

Aside from this finding, which can probably be overlooked in routine analyses, the present data indicate the reliability of Folch and Van Slyke's original procedure, method (d), for the determination of individual phospholipids (see also (3)). However, the results presented in Tables II and III have all been obtained with method (b) or (c).

Differences in the procedures for the purification of lipid extracts probably account for the different average values found in present (Table II, sixteen determinations) and previous analyses ((5), twenty determinations; (7), six determinations; (3), four determinations), the main difference being the lower values for cephalins.<sup>3</sup>

<sup>2</sup> It should be noted that in our hands Roman's method for choline sometimes gave errors as high as 10 per cent, whereas the analytical error of Tisdall's procedure for phosphorus never exceeded 2.5 per cent.

<sup>3</sup> According to the data of Thannhauser *et al.* (7) the proportion of sphingomyelins in human plasma is fairly constant (23 mg. per 100 cc.). If this figure is combined with those of the present analyses, lecithins would average 65, cephalins 20, and sphingomyelins 15 per cent of the total phospholipids in the plasma of normal fasting subjects.

TABLE I

*Phosphorus and Choline Content of Plasma Lipids Extracted and Purified by Different Methods*

The values are given in mg. per 100 cc. The methods are described in the text.

Sample No.	Phosphorus				Choline			
	Method							
	a	b	c	d	a	b	c	d
1	5.61	5.52	5.57	5.69	21.7	15.0	15.7	14.8
2	5.77	5.65			16.5	14.9		
3	6.30		6.38	6.48	30.0		19.4	18.3
4	7.39		7.30	7.55	24.8		23.0	22.0
5	5.65		5.75		21.7	18.2	17.6	
6	5.83		5.87	6.03	19.6		20.3	19.8
7	5.76	5.80	5.85		21.4	19.4	19.0	
8	6.05		6.27	6.92	20.1		17.4	16.9
9	6.17	5.90	5.95		17.5	16.8	17.2	
10	7.39			8.32	24.3			21.1
11			5.60	6.10			18.8	17.8
12	7.22		7.37	7.52	19.4		15.5	14.7
13			6.03	6.19			16.7	17.2

TABLE II

*Phospholipids and Total Fatty Acids Plus Unsaponifiable Substances in Normal Human Plasma*

	Cephalins	Choline phospholipids	Total phospholipids	Total fatty acids + unsaponifiable substances
Fasting; 16 analyses				
Averages, mg. per 100 cc....	30 ± 9.5	122 ± 14	152 ± 16	376 ± 72
Range, mg. per 100 cc....	13 - 46	103 - 157	128 - 191	245 - 498
Standard deviation, % ...	±31.6	±11.9	±10.8	±19.1
Extreme " %...	-57, +53	-16, +29	-16, +26	-35, +32
Absorptive; 10 analyses				
Averages, mg. per 100 cc....	47 ± 12	129 ± 17	176 ± 15	493 ± 74
Range, mg. per 100 cc.....	35 - 72	99 - 151	147 - 191	389 - 688
Standard deviation, %.....	±25.6	±13.1	±8.6	±15.0
Extreme " %.....	-26, +53	-23, +17	-16, +8.6	-21, +40

Except for the cephalin figures (which, being calculated indirectly, are probably less accurate), in the present investigations the mean and extreme deviations from the averages are moderate. They are much lower than in the extensive analyses of Kirk (5), in which individual deviations as great as 150 per cent for cephalins, 87 per cent for choline-containing phospholipids, and 90 per cent for total phospholipids were found.

Average values after ingestion of milk or cream (Table II) are higher than in the postabsorptive state, the increase of cephalins being most conspicuous. However, because of the extent of in-

TABLE III

*Phospholipids and Total Fatty Acids Plus Unsaponifiable Substances in Plasma of Men after Ingestion of Cream and without Cream*

The values are given in mg. per 100 cc.

Subject	Without or after cream	Cephalins	Choline phospholipids	Total phospholipids	Total fatty acids + unsaponifiable substances
J. F.	Without	35	106	141	301
	After	48	99	147	486
G. C.	Without	30	130	160	451
	After	35	150	185	525
J. H.	Without	35	103	138	415
	After	37	114	151	399
W. P.	Without	46	125	171	415
	After	40	141	181	490
P. C.	Without	34	157	191	485
	After	72	119	191	492
K. O.	Without	45	113	158	392
	After	64	120	184	502

dividual variations, the differences between the averages are not statistically significant.

If figures obtained on the same individual without and after ingestion of cream are compared (Table III), a proportionally greater rise of cephalins is found in three of the six experiments. Certainly no preferential increase of the choline-containing phospholipids is usually apparent in men absorbing milk fats.

On the contrary, an increase of both lecithins and total choline-containing phospholipids (with a simultaneous decrease of cephalins) frequently occurs in rabbits after ingestion of large amounts

of olive oil (1). Aside from the differences in the relative amounts and types of ingested fats, it may be noted that the slow absorption in herbivorous animals probably made the condition of our previous experiments similar to the continuous feeding of fats over a prolonged period.

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#### SUMMARY

Comparative data on the phosphorus and choline content of plasma lipids extracted and purified by different methods are reported.

Sixteen determinations on fasting young men gave the following average values, total phospholipids  $152 \pm 16$ , choline phospholipids  $122 \pm 14$ , cephalins  $30 \pm 9.5$  mg. per 100 cc. of plasma.

Ten determinations on human plasma after ingestion of milk or cream gave higher average values, especially for cephalins. No preferential increase of choline phospholipids has been demonstrated.

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# THE ACTIVITY OF CARBONIC ANHYDRASE IN RELATION TO THE SECRETION AND COMPOSITION OF PANCREATIC JUICE

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The concentration of the bicarbonate ion in pancreatic juice is 5 to 6 times that of the blood serum of the same animal when the rate of juice formation is near its maximum. As the rate of secretion diminishes, the amount of bicarbonate in the juice may fall to values of the same magnitude as for the serum (1, 2, 13). One possible explanation of these facts is that the bicarbonate of the juice arises from the carbon dioxide produced by the metabolic activity of the gland itself. Thus the bicarbonate content of the juice would be greatest when the activity of the gland, and presumably its carbon dioxide production, is at a maximum. Such a conversion of metabolic carbon dioxide into bicarbonate ions would, however, necessitate the rapid hydration of carbon dioxide by the pancreatic cells. According to van Goor (11), the pancreas is well supplied with the enzyme carbonic anhydrase which catalyzes just this reaction. Van Goor postulates (12) that carbonic anhydrase is present in the pancreas because it plays a rôle in pancreatic juice formation. Now, if such a mechanism is concerned in the formation of the bicarbonate of pancreatic juice, then changes in the activity of carbonic anhydrase should be reflected in the composition of the juice. With diminishing activity of this enzyme, one might expect a corresponding fall in the bicarbonate content of the juice with or without a corresponding decrease in the rate of juice formation. The activity of carbonic anhydrase may be inhibited by sulfanilamide (Mann and Keilin

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(15)) or by thiocyanate (Davenport (8)). A study, therefore, of the composition of pancreatic juice obtained from dogs before and after the injection of sulfanilamide or thiocyanate should furnish an answer to the rôle of carbonic anhydrase in pancreatic juice formation. Such a study is reported here.

### *Methods*

Dogs which had not been fed for 24 hours were used in all experiments. The weight of these animals varied from 8 to 22 kilos (average, 15 kilos). Nembutal (32 mg. per kilo) injected into the peritoneum was used as an anesthetic. A cannula was inserted in the main pancreatic duct by a procedure similar to that reported by Ball (1). Secretin prepared by the method of Cowgill and Mendel (6) was injected into the left femoral vein to stimulate the flow of pancreatic juice which was collected under oil in graduated 15 cc. centrifuge tubes. Secretin injections were given at regular intervals and at dosage levels that insured as maximum a rate of flow of juice as was possible. The first flow of juice was considered to be a composite of that stimulated by the secretin injection and that already present in the gland. A second juice sample which always showed an increase in bicarbonate content was used to represent the control juice. A solution of sulfanilamide or sodium thiocyanate was then injected into the right femoral vein from a burette attached to a needle. Secretin was again injected and a sample of juice obtained. A blood sample, collected under oil, was taken from the jugular vein. Serum was drawn off under oil after the blood had been centrifuged. In two of the experiments in which sodium thiocyanate was used, the total dosage was increased by further injections, after each of which the juice flow was stimulated by secretin, and samples of juice and blood were obtained. In all other experiments, in which the total dosage of sulfanilamide or sodium thiocyanate was administered in one injection, samples of juice and serum were collected at definite intervals following secretin injections. The dog was maintained under the influence of the nembutal throughout the experiment, and sacrificed at the end.

The total  $\text{CO}_2$  content of the pancreatic juice and serum samples was determined by the method of Van Slyke and Neill (20). The analysis for chloride was made according to the method of Van

Slyke as modified by Wilson and Ball (21). When thiocyanate was present, it was shown by experiment that it was destroyed quantitatively during the acid digestion, and did not affect the accuracy of the chloride determination.

Hydrogen ion concentrations of the juice were determined potentiometrically at 30° by means of a glass electrode of the type introduced by MacInnes and Belcher (14). An amplification unit similar to that described by van Dyke (9) was employed. Calibration of the glass electrode was performed with solutions of known pH, determined with the hydrogen electrode provided with a Clark type of shaking vessel and a saturated KCl-calomel half-cell standardized against both a 0.1 M KCl-calomel half-cell and standard acetate buffer, as described by Clark (5). Clark's tentative standard of potential was used.

The concentration level of sulfanilamide obtained in the samples of juice and serum was determined by the method of Bratton and Marshall (3). Since this drug is not acetylated in the dog (16), preliminary hydrolysis was unnecessary to obtain total sulfanilamide concentrations.

Analysis for thiocyanate concentration in juice and serum was made according to the method of Crandall and Anderson (7).

### *Results*

Table I summarizes the results of a typical experiment in which sulfanilamide was injected. Tests on Juice 2 collected before the injection showed that pancreatic juice contained no substances reacting with the sulfanilamide reagent. The data show that neither the composition of the pancreatic juice nor its rate of secretion undergoes any significant change as a result of the injection of this drug. Sulfanilamide appeared promptly in the juice but its concentration in the juice in this experiment did not reach values comparable to that of the blood serum until several hours after the injection. Similar differences in sulfanilamide concentrations in blood and pancreatic juice have been reported by Marshall, Emerson, and Cutting (17) and by Carryer and Ivy (4). The latter workers have also reported no alteration in rate of juice secretion when sulfanilamide concentrations in the juice reached a value of 13 mg. per cent.

A sulfanilamide concentration of 20 mg. per cent was obtained



in Juice 3 collected immediately after the injection. This value corresponds to a concentration of  $1.2 \times 10^{-3} M$ , which is 60 times the value which Mann and Keilin (15) found to inhibit almost completely carbonic anhydrase in their experiments.

In Table II, the results of a typical experiment in which sodium thiocyanate was injected are given. Juice 2, collected before the injection, gave no reaction with the thiocyanate reagent. Four injections of sodium thiocyanate were made in all, serum and juice samples being collected after each injection. Thiocyanate

TABLE I

*Composition of Pancreatic Juice and Blood Serum As Influenced by Injection of Sulfanilamide*

Dog 3, male, 13.4 kilos.

Sample	Time	Amount of sample	pH	Total CO <sub>2</sub>	Cl	Sulfanilamide
		cc.		mm per l.	mm per l.	mg. per cent
Juice 1	10.45-11.06 a.m.	7	8.10	127	31	
" 2	11.06-11.27 "	9	8.35	135	22	0
	11.27-11.49 "	135	1.5% sulfanilamide solution injected intravenously			
" 3	11.49 a.m.-12.10 p.m.	8.3	8.35	124	27	20
Serum 1	11.50 "	20*		24	106	32
Juice 4	12.10-12.32 p.m.	7	8.40	127	25	13
Serum 2	12.32 p.m.	20*		24		25
Juice 5	2.09-2.35 p.m.	6.5	8.40	120	30	10
Serum 3	2.43 p.m.	11*		24		12

\* Amount of whole blood.

appeared promptly in the juice after intravenous injection. No appreciable changes in the chloride or bicarbonate composition of juice or serum occurred after the first two injections. After the third injection, the rate of juice secretion declined and there was a slight fall in bicarbonate, and a concomitant rise in chloride concentration. A fall in serum chloride but not bicarbonate concentration also became evident. After the last injection, the animal showed evidence of marked distress, with violent abdominal heaving and diarrhea. A response was, however, still obtained on injection of secretin, but the rate of secretion had markedly

diminished. The fall in bicarbonate and rise in chloride concentration which occurred in Juice 6 we believe can be attributed entirely to the altered rate of secretion. This latter effect has been discussed in the introduction. The concentration of thiocyanate in Juice 6 of 28 mm per liter is 4 times the concentration Davenport

TABLE II  
*Composition of Pancreatic Juice and Blood Serum As Influenced by  
Injection of Sodium Thiocyanate*

Dog 11, male, 16 kilos.

Sample	Time	Amount of sample  cc.	pH	Total CO <sub>2</sub>	Cl	Thio- cyanate
				mm per L.	mm per L.	mm per L.
Juice 1	10.15-10.25 a.m.	8	8.0	122	30	
" 2	10.25-10.36 "	11	8.15	137	22	0
Serum 1	10.32 a.m.	20*		24	109	
	10.36-10.49 a.m.	80	Sodium thiocyanate in- jected†			
Juice 3	10.52-11.02 "	6.5		130	26	8
Serum 2	10.54 a.m.	20*		24	103	21
	11.05-11.13 a.m.	40	Sodium thiocyanate in- jected†			
Juice 4	11.17-11.27 "	8	8.10	134	23	12
Serum 3	11.20 a.m.	20*		25	99	28
	11.29-11.39 a.m.	40	Sodium thiocyanate in- jected†			
Juice 5	11.42-11.52 "	4	8.05	127	30	16
Serum 4	11.50 a.m.	20*		24	93	35
	11.52 " -12.04 p.m.	40	Sodium thiocyanate in- jected†			
Juice 6	12.08-12.35 p.m.	3		108	43	28
Serum 5	12.30 p.m.	12*		20	89	42

\* Amount of whole blood.

† 10 per cent solution intravenously.

(8) reports as necessary for the nearly complete inhibition of carbonic anhydrase.

A summary of the pertinent data of all experiments performed is given in Table III. Each experiment summarized here resembled in detail those presented in either Tables I or II. Four experiments were performed in which sulfanilamide was injected,

the total dosage varying as indicated. In no instance was a change in the rate of secretion or the bicarbonate concentration of the juice greater than 10 per cent produced.

TABLE III  
*Summary of Experiments*

Compound injected	Dog No.	Total dosage	Rate of pancreatic juice flow	Maximum drug concentration obtained		Total CO <sub>2</sub>	
				Pancreatic juice	Serum	Pancreatic juice	Serum
		gm. per kg.	cc. per min.	mg. per cent	mg. per cent	mM per l.	mM per l.
Sulfanilamide	1	0	0.61			110	
		0.005	0.67			119	
	2	0	0.33			146	
		0.1	0.33	12	14	131	
	3	0	0.45			135	
		0.15	0.43	20	32	124	24
	4	0	0.13			92	
		0.5*	0.46	64	77	93	33
				mM per l.	mM per l.		
Sodium thiocyanate	5	0	0.44				
		1.5†					
	6	0	0.33			116	
		1.2	0.044	19	43	91	16
	8	0	0.28			135	
		1.0	0.011	21	43	94	21
	9	0	0.95			132	
		0.25	0.70	5	13	115	25
		0.50	0.55	9	20	110	25
		1.00	0.00		40		20
	11	0	1.1			137	24
		0.5	0.65	8	21	130	24
		0.75	0.80	12	28	134	25
		1.00	0.40	16	35	127	24
		1.25	0.11	28	42	108	20

\* Injected as the sodium salt.

† The dog died as a result of the injection.

Five experiments were performed in which sodium thiocyanate was injected. In one of them, a dosage of 1.5 gm. per kilo proved fatal. In the other four experiments, a total dosage was administered which produced obvious distress in the animal. In two of these cases, Dogs 6 and 8, the thiocyanate was administered step-

wise, as outlined in Table II; and in the other two experiments, Dogs 9 and 11, the injection was uninterrupted. In all experiments, the results are similar. Injection of thiocyanate caused a decrease in rate of flow which was attended by a diminution in bicarbonate content.

#### DISCUSSION

The results presented here are interpreted by us to indicate that carbonic anhydrase is not essential for the formation of the bicarbonate of pancreatic juice. We feel that the experiments in which sulfanilamide was injected furnish the best evidence for such a conclusion. In the case of Dog 4, Table III, there was no alteration in juice composition when the concentration of sulfanilamide in the juice reached a value nearly 200 times that described by Mann and Keilin (15) as inhibiting almost completely carbonic anhydrase in their experiments. Since there is no indication that the pancreas concentrates sulfanilamide in the juice, the amount of this drug within the cells of the pancreas is probably not greatly different from that found in the juice secreted by these cells. Thus, the carbonic anhydrase in these cells should have been sufficiently inhibited so that the composition of the pancreatic juice would have reflected this alteration in enzyme activity, if this enzyme was essential for the formation of juice bicarbonate.

The results of the thiocyanate injections are perhaps not as conclusive, but they tend to support the results of the sulfanilamide injections. When concentrations of thiocyanate in the juice reached values twice those described by Davenport (8) as nearly completely inhibitory for carbonic anhydrase, little alteration in juice composition was observed. To obtain higher juice concentrations such massive doses of thiocyanate are needed that general toxic effects appear which tend to obscure the interpretation of the results. Even under such conditions, however, no striking alterations in juice composition were observed.

Now since the observations of Mann and Keilin (15) and Davenport (8) on the inhibition of carbonic anhydrase were made on enzyme preparations obtained from red blood cells or stomach mucosa, a valid criticism of our conclusions might be that the carbonic anhydrase of the pancreas is of a quite different nature and is not inhibited by such agents. We have, therefore, prepared extracts from dog pancreas and studied their carbonic an-

hydrase activity by a method similar to that of Philpot and Philpot (18). By this method the time required for conversion of carbon dioxide into bicarbonate is measured. Sulfanilamide inhibited the carbonic anhydrase activity of these extracts at the same concentrations that were required to inhibit enzyme preparations made by us from gastric mucosa or red blood cells. These inhibiting concentrations also agreed well with those reported by Mann and Keilin.

It is of interest to compare the action of sulfanilamide and thiocyanate upon pancreatic secretion with their action on gastric juice secretion. Davenport (8) found that thiocyanate administration produced a marked decrease in the output of gastric juice. He suggested these results might be interpreted as due to inhibition of the carbonic anhydrase known to be present in gastric mucosa, and postulated that this enzyme was concerned in the mechanism whereby HCl was formed in the stomach. We have observed a similar decrease in rate of secretion of pancreatic juice after administration of thiocyanate. Feldberg, Keilin, and Mann (10) were able to confirm Davenport's observations with thiocyanate. They, however, also administered sulfanilamide and found that it was without effect upon gastric juice secretion. The conclusion was, therefore, reached that carbonic anhydrase was not necessary for the production of gastric juice. These findings and conclusions agree with ours for the effect of sulfanilamide on pancreatic juice formation. Thus, it would appear that the presence of carbonic anhydrase in stomach and pancreas cannot be explained on the basis that it plays an essential rôle in the formation of the juices of these glands. Are these glands storehouses or sites of production for carbonic anhydrase? In the case of the pancreas, the isolation of insulin and carbonic anhydrase as proteins with high zinc content and their joint occurrence in this gland are perhaps suggestive.

We feel that the experiments reported here do not serve to answer the question as to the source of the bicarbonate found in pancreatic juice. They suggest, however, that metabolic carbon dioxide from the gland itself is not concerned and that juice bicarbonate may be derived from the blood. In this connection, a calculation of the metabolic rate that would be demanded of the pancreas if all the bicarbonate of the juice was derived from the metabolic carbon dioxide of the gland is of interest. The follow-

ing experiment furnished the necessary data for such a calculation. Cannulas were introduced into both pancreatic ducts of a dog weighing 11 kilos and secretion of juice stimulated at a maximum rate by secretin injection. The rate of juice flow was 1.25 cc. per minute and the total carbon dioxide concentration of the juice was 125 mm per liter. At the end of the experiment, the pancreas was removed and found to weigh 31 gm. From these values it can be calculated that the equivalence of 0.112 cc. of carbon dioxide gas is secreted in the juice every minute for each gm. of wet pancreas tissue. Still, Bennett, and Scott (19) reported that the secreting pancreas consumed 0.062 cc. of  $O_2$  per minute per gm. of wet tissue and had an R.Q. of 1.05. This corresponds to a carbon dioxide output by the gland of 0.065 cc. per minute per gm. of wet tissue, a value which is about half that necessary to supply the quantity of carbon dioxide found in the juice sample under consideration. The output of juice carbon dioxide may be also expressed in terms of  $Q_{CO_2}$ . If the water content of pancreas is taken as 74 per cent, an average value in our determinations, and it is assumed that all carbon dioxide of the juice is metabolic in origin, the  $Q_{CO_2}$  of pancreas would be 26. With an R.Q. of 1.00, this corresponds to a  $Q_{O_2}$  of 26, which is a value not far from the maximum reported in the literature for any tissue. Thus, though it is not impossible for the metabolic  $CO_2$  to furnish all the bicarbonate found in this juice sample, it would certainly require the total carbon dioxide output of the gland for this purpose. In view of these facts and the failure of sulfanilamide to alter the composition of the juice, we are inclined to agree with the conclusion of Still, Bennett, and Scott (19) that at least part of the juice bicarbonate arises from the bicarbonate of the blood.

#### SUMMARY

The activity of the carbonic anhydrase of pancreas is shown to be inhibited by concentrations of sulfanilamide similar to those reported by previous workers for preparations of this enzyme obtained from other tissues.

Injections of sulfanilamide into dogs so as to produce concentrations of this drug in pancreatic juice 200 times the minimum concentration necessary to inhibit carbonic anhydrase were without effect on the secretion rate or composition of the juice.

Thiocyanate injections gave similar results when the dosage

was just sufficient to produce concentrations of thiocyanate in the juice which were equal to the minimum inhibitory concentration for carbonic anhydrase. Higher dosage produced a general toxic effect and a decrease in rate of juice secretion.

The conclusion is reached that carbonic anhydrase plays no essential rôle in the production of the bicarbonate of pancreatic juice.

One of us (H. F. T.) wishes to express appreciation to Professor A. Baird Hastings for his courtesies in providing laboratory facilities and helpful cooperation during her stay in this department.

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# A MODIFIED HALDANE GAS ANALYZER FOR ANALYSIS OF MIXTURES WITH ONE HUNDRED PER CENT ABSORBABLE GAS

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Some years ago a need arose for a gas analyzer that would allow the concentrations of acetylene and oxygen to be raised above those which can be handled in the ordinary type of machine. The present analyzer was designed for this purpose. In determinations of cardiac output by the Grollman method, the presence of higher concentrations of either acetylene or oxygen or of both was not found to affect the estimate significantly.<sup>1</sup> The special analyzer was not therefore described. With the recent intensified importance of the desaturation of the body with nitrogen before flying at great heights, the determination of the rate of nitrogen elimination from the body attains greater importance. For such determinations, as well as for the exact estimation of alveolar air concentrations in individuals breathing high concentrations of oxygen, the apparatus here described has a special usefulness. For this reason a description is now submitted. The apparatus is so designed that it can be utilized for many types of analysis. For the analysis of expired air an accuracy can be attained that is several times greater than that obtainable with the ordinary Haldane analyzer.

*Apparatus*—The principles involved are throughout those developed by Haldane. The main burette in which the gas being analyzed is contained is, however, double; the two parts of which it is composed are connected by a T-stop-cock. The larger of

<sup>1</sup> Bazett, H. C., Scott, J. C., Maxfield, M. E., and Blithe, M. D., *Am. J. Physiol.*, **116**, 551 (1936).



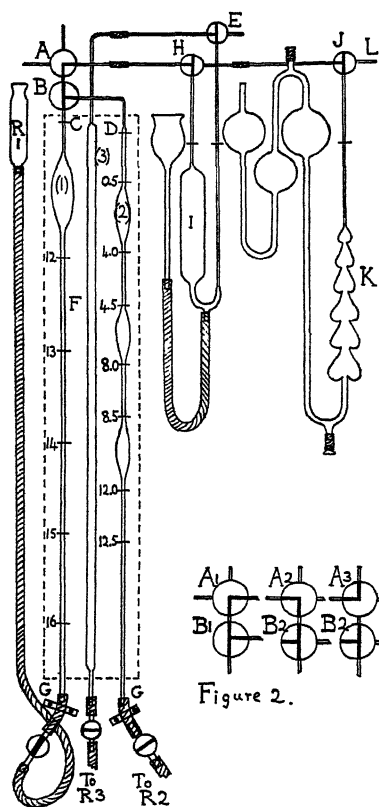


Figure 1.

FIG. 1. The two parts of the double burette (1) and (2) are shown diagrammatically within their water bath *F*. The numbers on the tubes indicate the graduated sections (graduated in 0.01 ml.) with zero positions at *C* and *D* respectively. Burette (1) is made of tubing of 3.5 mm. bore and (2) of tubing of 3.2 mm. bore. The upper tubing connecting to the cocks *A* and *B* as well as that connecting to the absorbers is of 1.5 mm. bore. Between (1) and (2) lies the control burette (3) consisting of tubing of 5 to 6 mm. bore. All three burettes are connected below with their reservoirs *R1*, *R2*, and *R3* by rubber tubing with cocks intervening. On the tubing connecting to burettes (1) and (2) are screw clamps *G* to effect fine adjustments. Cock *E* connects the control system to the room when required. Cocks *H* and *J* connect to the  $\text{CO}_2$  absorber *I* and the oxygen absorber *K*. They may be of the *T*-type as shown or may have double oblique bores. Tube *L* beyond cock *J* serves for cleaning or for connecting a third absorber. Rubber tubing connections are shown by shading.

FIG. 2. Various combinations of the positions of cocks *A* and *B* are indicated.

these two burettes (labeled (1) in Fig. 1) contains 16 ml., 12 ml. in the bulb and 4 ml. in the graduated tube below it. It alone is used to pump the gas back and forth. The smaller (labeled (2) in Fig. 1) consists of three bulbs each of 3.5 ml. with a graduated section of tube of 0.5 ml. capacity on either side of it. It can be employed to contain the gas being analyzed or to store, under standardized conditions, nitrogen or other unabsorbable gas from a previous analysis.

The apparatus may be employed in three different ways: (1) Stop-cock *B* may be placed in position *B2* (see Fig. 2), thus disconnecting the smaller burette; the larger may then be used as an ordinary Haldane analyzer. In this way volumes of about 16 ml. with absorbable gases amounting to 25 per cent of the total can be analyzed. The analysis can be made with the same speed and precision as in the ordinary Haldane analyzer. (2) The stop-cock *B* may be placed in position *B1*; excess nitrogen may be expelled from both the larger and smaller burettes, which can then both be filled with the unknown gas. The volume thus taken for analysis can be as large as 28.5 ml., with a consequent gain in accuracy though with a loss in speed. Such analyses can be made provided that 12 ml. of the total are ultimately left unabsorbed. Therefore mixtures containing over 57 per cent of absorbable gas can be analyzed. (3) The third method is that for which the apparatus was primarily designed. Following an analysis of air, 12.5 ml. of residual nitrogen are transferred to the smaller burette (2) in which the nitrogen is stored to assist in the following analysis. The volume so stored is carefully balanced against the control tube (3) in the ordinary way immediately before any further excess of nitrogen is ejected. In such a balancing cock *B* is obviously in position *B1*. When balance is attained, cock *B* is first turned to position *B2*; then and then only is cock *A* turned to position *A2*, the excess nitrogen is ejected, and the new gas introduced. When the new gas has been introduced, cock *A* is returned to position *A1* and cock *B* to position *B1* before the volume of the gas introduced is read. During the analysis the nitrogen stored in burette (2) is added as required to that in burette (1). Consequently, any gas of any volume up to 16 ml. can be manipulated, even if 100 per cent of the gas is absorbable. It is often convenient to be able to analyze a sample below the

normal volume (e.g., of 8 ml.). This also can be done without modifying the procedure, though naturally with some loss of accuracy.

*Apparatus Utilized As a Single Burette*—The arrangement may best be seen in Fig. 1. The entrance cock through which the gas is introduced (*A*) is of the type used by Grollman. At the end of an analysis this cock is in position *A1* (see Fig. 2); it is turned to *A2* to allow the discharge of excess nitrogen; it is then turned to *A3* to allow the connecting tubing to be washed out with the gas to be analyzed. It is returned to position *A2* for the introduction of the gas and to *A1* for analysis. If the lower cock *B* be retained in position *B2*, then burette (*1*) may be utilized as an ordinary Haldane analyzer and all analyses proceed in the orthodox manner. When the nitrogen is discharged, the mercury meniscus is brought to the zero mark at *C*; above it is 1 per cent sulfuric acid sufficient to reach almost to the side branch of the cock *B*, but not beyond it. The gas introduced is equal to the volume from the zero graduation to the ultimate position on the scale, plus the volume contained in cock *A*. This cock originally contained nitrogen but finally contains the gas under analysis. The precautions to be taken in relation to the control system are described later.

When the single burette is used in this manner as an ordinary Haldane apparatus, analysis of outside air, expired air, and the like can be conducted with speed and accuracy. There is some gain in accuracy from the larger volume utilized and some loss through the complications of the dead end of the T-cock *B*. A typical set of successive analyses of outside air in triplicate by this technique is given in Table I in Section A.

*Use of Both Burettes to Accommodate Unknown Gas*—In this case the introduction of the gas proceeds as before but cock *B* is in position *B1* throughout, and nitrogen is expelled also from burette (*2*). The mercury meniscus in the smaller burette is also brought to the zero mark *D*; 1 per cent sulfuric acid fills the top part of the burette up to, but not into, stop-cock *B*. The total volume is taken into both burettes (up to a volume of 28.5 ml.). In gas absorption the mercury in burette (*1*) is alone used as a pump. The gas in burette (*2*) is merely forced up by the mercury to join that in the other burette. When the connecting tubes

are washed out when the gas is being fetched from the carbon dioxide absorber and the like, the absorption of gases may be speeded by reversing the columns of gas contained in the two burettes. Thus the last gas from the oxygen absorber may be taken into burette (2) which will thus contain pure nitrogen, if air is being analyzed. When the gas is returned to the ab-

TABLE I  
*Consistency Obtainable in Successive Analyses in Triplicate*

Gas analyzed		Volumes utilized	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>
		<i>ml.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Outside air	A. With burette (1) only	15.944	0.04	20.91	79.05
		15.933	0.04	20.94	79.02
		15.980	0.03	20.94	79.03
		Means.....	0.037	20.930	79.033
	B. With both burettes for unknown gas	28.504	0.035	20.935	79.030
		28.420	0.039	20.929	79.032
		28.474	0.039	20.935	79.026
		Means.....	0.038	20.933	79.029
	C. With burette (2) to store N <sub>2</sub>	15.969	0.04	20.93	79.03
		16.015	0.04	20.95	79.01
		15.974	0.05	20.91	79.04
		Means.....	0.043	20.930	79.027
Tank oxygen	D. With burette (2) to store N <sub>2</sub> under steady room conditions	15.925	0.04	99.42	0.54
		15.929	0.03	99.44	0.53
		15.958	0.02	99.45	0.53
		Means.....	0.030	99.437	0.533

sorber, the gas from burette (1) may be passed first, and the pure nitrogen in burette (2) be used to wash it further into the absorber.

The accuracy thus obtainable in the analysis of air is illustrated by the successive analyses in triplicate given in Section B of Table I. An analysis takes about 15 minutes.

*Use of Second Burette As a Store for Nitrogen*—In the third

method of use nitrogen at the end of an analysis is moved from the bulb of burette (1) into the CO<sub>2</sub> absorber and then is taken into burette (2). Any excess nitrogen is taken into the bulb of burette (1). (Great care must be used for the balancing point may be at a position where the mercury is in the center of the larger bulb.) Cock *E* on the control tube is then opened to the air and the control system is brought precisely to atmospheric pressure, while the gases in the two burettes are balanced against it. Cock *E* is then closed, the balance is rechecked, and minor adjustments are made if needed. Cock *B* is then turned from *B1* to *B2*, then cock *A* from *A1* to *A2*, and the excess nitrogen is ejected as usual. When the new gas has been introduced and cock *A* has been returned to position *A1*, cock *B* is returned to the *B1* position before balances are attained and volumes read. When these readings are made, care must be taken to insure that there is no water-lock produced by a bubble in cock *B*. Such a lock is readily formed if the 1 per cent sulfuric acid in either burette is slightly in excess and reaches the branching of the T-cock. If the machine is reasonably clean, such a lock, if formed, can readily be broken. After the volume is read, the gas in burette (1) is absorbed, and nitrogen is added to it from burette (2) as required. The nitrogen may be used to wash the absorbable gas into the absorbing solution. The pumping action in burette (1) may be conducted with cock *B* in either position *B1* or *B2*, according to the analysis being made. If the cock is in position *B1*, the mercury in the burette should be at the zero position.

The accuracy obtainable with such procedures is indicated in the successive triplicate analyses of outside air shown in Section C of Table I and of tank oxygen shown in Section D of Table I. The greater accuracy is normally obtainable with the lower percentages of absorbable gas. The slightly greater accuracy shown in Table I for analysis of tank oxygen is due to the analyses having been conducted when the room temperature was particularly stable, while those for outside air were made under ordinary conditions. As will be explained, analyses of pure oxygen (or of carbon dioxide) are particularly liable to errors due to changes in temperature, and for maximal accuracy temperature fluctuations should be kept small. In spite of this, under ordinary conditions

errors should never exceed  $\pm 0.05$  per cent. The time consumed in using the analyzer in this way is also about 15 minutes per analysis.

*Technical Details*—The difficulties and pitfalls will be readily recognized by those familiar with gas analysis. The compensation for temperature changes attainable from the dummy burette is only accurate if the ratio of the gas contained in the tubes within the water bath to that contained in tubes in air in the active system is the same as the ratio of these two sets of tubes in the control system. Obviously these conditions cannot be met exactly, since the ratio of the gas contained in the tubes in the water bath to that in the connecting tubes in the active system is constantly changing during the course of an analysis. The larger the proportion of absorbable gas, the greater is this change and the less accurate is the compensation attained. The maximal compensation will be attained if the ratios are comparable during the relatively slow process of the removal of the last traces of oxygen. For this reason the control burette (3) is made of simple tubing of 5 to 6 mm. bore and is fitted below with a stop-cock and a mercury reservoir. When the machine is standardized, not only are the various calibrations exactly checked but also the optimal positions are determined for the water meniscus above the mercury in this control tube to attain compensation for any given volume in the active burettes. This is done empirically by setting the water level in the control burette (3) opposite some given position on burette (1) and determining the apparent change in volume of burettes (1) and (2), when the water bath is either cooled or warmed. These determinations are made with various volumes in burettes (1) and (2). The optimal position for the fluid level in the control system is thus determined empirically; such determinations made on five or six different volumes in the active burette allow a curve to be drawn. The control tube can then be set at its optimal position for any given set of analyses. Even so, if the changes in volume are great (as in analysis of tank oxygen), compensation attainable towards the end of the analysis implies that it is inadequate at the start and that estimates of  $\text{CO}_2$  concentrations may be slightly in error. (It may be noticed in Section D of Table I that the discrepancies were regular and progressive—

their direction could be predicted from the direction of the slight temperature changes.) The adjustable character of the control tube is a definite advantage and one which can readily be incorporated in any Haldane analyzer.

Anyone undertaking the use of an analyzer of this type should be warned to be particularly careful if precision is needed in the analysis of pure oxygen. Under such conditions there is little or no nitrogen available to expel through cock *A* before the new sample is taken in. It is consequently imperative that this process be carried out very carefully, so that the connecting tubes between the burette and the exterior do not get prematurely fouled by the incoming air. At the time cock *A* is turned to position *A2* the pressure in burette (*1*) must be slightly greater than atmospheric and a slight amount of nitrogen must be available for expulsion. Even when larger volumes of nitrogen are available, the system should be carefully balanced against real atmospheric pressure before cock *E* is closed and nitrogen is expelled, as already explained. There is a volume of gas existing between the zero point *C* (or in the second method from zero points *C* and *D*) and cock *A*, consisting of nitrogen maintained in the system and measured against the balance of control burette (*3*); yet when cock *A* is open, this gas can freely escape if the pressures within and without the system are not the same. The volume of this gas is small; it can be reduced to negligible proportions by using fluid above the mercury adequate to reach up to but not into cock *A*. If cock *B* is kept exceptionally clean, this may be done without difficulty, but if cock *B* becomes greasy, troublesome water-locks readily form.

In standardization of the calibrations particular care must be taken to use the burettes wet and to move the mercury as it is moved during an analysis. The water in burette (*2*) tends to collect in the lower bulbs and to rise to the top only slowly if the mercury is brought up rapidly in this irregular system. In use the movements of the mercury should be controlled by the cock so that the mercury only rises at a moderate speed. Standardization should be made with the same rates of movement.

The ease of handling and the accuracy of the machine are obviously improved if the volume between cocks *A* and *B* is as small as possible.

I would like to pay tribute to the skill and care of Mr. James D. Graham, of the University of Pennsylvania, who built the two cocks with a length of only 6 mm. of capillary tubing between them.

#### SUMMARY

A modification of the Haldane gas analysis apparatus is described. This modification is adapted to allow analysis of samples of gas up to 16 ml. from mixtures containing up to 100 per cent of absorbable gas. This is attained by using a main burette of 16 ml. with 4 ml. graduated in 0.01 ml., and a second burette connected to it by a T-cock containing 12.5 ml. The latter burette may be used to store nitrogen, which can be accurately measured and be utilized to make up the volume in the main burette as gas is absorbed. The smaller burette consists of three sections with a bulb of 3.5 ml. and a graduated tube above it of 0.5 ml. Below the lowermost bulb an additional 0.5 ml. is graduated. The accuracy obtainable is of the same order as that of the ordinary Haldane apparatus.

The double burette can be used not only in this way but also both burettes may be used to contain the unknown gas. Samples of gas up to 28.5 ml. in volume may thus be analyzed if the absorbable gas is 57 per cent or less of the total. The accuracy obtainable in this form in analysis of outside air is of the order of  $\pm 0.005$  per cent.

If desired, the main burette alone may be used exactly as an ordinary Haldane analyzer.

Owing to the very variable volumes that may be utilized in the active burette, it is important to have the volume of air in the control burette adjustable. The arrangement for so doing is described, and it is pointed out that it is a useful addition to the ordinary Haldane analyzer.





# MECHANISM OF SYMBIOTIC NITROGEN FIXATION

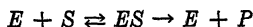
## V. NATURE OF INHIBITION BY HYDROGEN\*

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(Received for publication, December 9, 1940)

In a previous communication (1) several types of evidence were presented which strongly indicated that molecular hydrogen was a specific inhibitor of symbiotic nitrogen fixation. For interpretation of the mechanism of this biological reaction the question of the nature of the inhibition, whether competitive or non-competitive, must be answered. If the inhibition is non-competitive, it will be essentially independent of the  $pN_2$ , but if competitive both the  $pH_2$  and the  $pN_2$  will be concerned. Of the several technical devices which test this point, those based on the equation developed by Lineweaver and Burk (2) are probably the most accurate and conclusive. These workers showed that if the reactions



be assumed, Equation 1<sup>1</sup> can be derived.

$$\frac{1}{v_i} = \frac{1}{V} \left[ K_i + \frac{K_i(I)}{K_i} \right] \frac{1}{(S)} + \frac{1}{V} \quad (1)$$

In Equations 1 to 6 the following symbols are used.

\* Herman Frasch Foundation in Agricultural Chemistry, Paper No. 211. Technical assistance on the experiments was supplied in part by workers employed in the University's Works Progress Administration, Natural Science Project.

<sup>1</sup> For details of the derivation see Elvehjem, Wilson, *et al.* (3).

- $v$  = velocity of reaction in absence of inhibitor  
 $v_i$  = velocity of reaction in presence of inhibitor  
 $(S)$  = concentration of substrate  
 $(I)$  = concentration of inhibitor  
 $(E)$  = concentration of enzyme  
 $P$  = product of reaction (usually the measured quantity)  
 $V$  = maximum velocity when enzyme is saturated with substrate  
 $K_s$  = dissociation constant of enzyme-substrate complex  
 $K_i$  = dissociation constant of enzyme-inhibitor complex

The velocity equation for the reaction in the absence of the inhibitor is

$$\frac{1}{v} = \frac{1}{V} \left[ \frac{K_s}{(S)} + 1 \right] \quad (2)$$

Comparison of Equations 1 and 2 shows that the effect of an inhibitor is to increase the slope of the line obtained when the reciprocal of the velocity is plotted against the reciprocal of the substrate concentration. If inhibition is competitive, the term  $[K_s(I)/K_i]$  in Equation 1 exists, and the slope of the line increases; if non-competitive, the slope is unchanged in the presence of inhibitor, but the intercept is increased ( $V$  is lowered).

A second method for detection of the type of inhibition depends on the following considerations. From the terms in Equation 1

$$\frac{1}{v_i} = \frac{K_i K_s + K_s(I) + (S)K_i}{V(S)K_i} \quad (3)$$

$$= \frac{K_s + (S)}{V(S)} + \frac{K_s(I)}{V K_i(S)} \quad \text{but } v = \frac{V(S)}{K_s + (S)} \quad \text{or } V(S) = v[K_s + (S)] \quad (4)$$

Then

$$\frac{1}{v_i} = \frac{1}{v} + \frac{K_s}{K_i} \cdot \frac{(I)}{v[K_s + (S)]} \quad (5)$$

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i} \cdot \frac{(I)}{K_s + (S)} \quad (6)$$

Consideration of Equation 6 shows that if  $[v/v_i]$  is plotted against  $(I)$  for two different values of  $(S)$ , the resulting lines will have significantly different slopes with an intercept of 1.00 for competitive inhibition and will coincide if the inhibition is non-competitive.

The choice of method for testing the nature of inhibition depends largely on whether it is more advantageous to hold constant the concentration of inhibitor or substrate. This, in turn, depends on which will allow the greatest number of values to be assigned to the dependent variable. For inhibition of symbiotic nitrogen fixation by hydrogen, both methods have been used, but more points for the establishment of the line can be obtained if the  $pN_2$  is held constant and the  $pH_2$  taken as the dependent variable (Equation 6).

### Methods

Details of the methods have been given in previous reports. Briefly, clover plants are grown in a closed system under a constant  $pO_2$  but with varying partial pressures of nitrogen and hydrogen. Usually the total pressure of the gases did not equal unity, and the plants were accordingly grown under a partial vacuum; in Experiments 6 and 7 helium was added to bring the total pressure to 1 atmosphere in all cases. In Experiments 1 to 3 two harvests only were made and the velocity of fixation calculated from the formula

$$k = \frac{2.303}{\text{time in days}} \times \log \frac{\text{mg. N at harvest}}{\text{mg. N at start of treatment}} \quad (7)$$

In Experiments 4 to 7, four to five harvests were periodically taken and the slope of the line obtained by plotting *log mg. of N* against *time in days* was determined by the usual statistical procedures. This slope multiplied by 2.303 gives the "best" estimate<sup>2</sup> of  $k$ . Table I provides a comparison of  $k$  values calculated by the formula from the first and final harvest and those obtained from the regression line based on four harvests. The close agreement between corresponding values indicates that little error was introduced in those experiments in which  $k$  was deter-

<sup>2</sup> In previous reports of this series in which the advantages were emphasized of the use of the specific reaction constant of fixation for measurement of reaction velocity when the total amount of cell substance (and hence enzyme concentration) changes with time, the letter  $g$  has been used to designate the quantity defined by the formula. Since  $g$  is used in bacteriological literature for *generation time*, it appears desirable to use the original chemical symbol from which the formula was derived.

mined by two values only. The details of the individual experiments were as follows:

*Experiment 1*—Planted October 18, 1937; Harvest I, November 29; Harvest II, January 3, 1938.

TABLE I  
*Comparison of  $k$  Values Based on Lines of Regression and Formula (Experiment 4)*

Treatment		$k$ value based on formula	$k$ value based on line of regression
$p\text{H}_2$	$p\text{N}_2$		
<i>atmosphere</i>	<i>atmosphere</i>		
0.0	0.15	0.0519	0.0505
		0.0485	0.0473
	0.30	0.0497	0.0483
		0.0532	0.0522
	0.45	0.0522	0.0515
0.10	0.15	0.0487	0.0476
		0.0363	0.0345
	0.30	0.0390	0.0370
		0.0416	0.0415
	0.45	0.0435	0.0437
0.20	0.15	0.0446	0.0435
		0.0452	0.0450
	0.30	0.0312	0.0301
		0.0319	0.0318
	0.45	0.0344	0.0338
0.35	0.15	0.0357	0.0355
		0.0442	0.0433
	0.30	0.0452	0.0448
		0.0273	0.0262
	0.45	0.0298	0.0288
0.50	0.15	0.0333	0.0318
		0.0362	0.0352
	0.30	0.0354	0.0336
		0.0388	0.0376
	0.45	0.0142	0.0142
		0.0163	0.0169
		0.0263	0.0257
		0.0267	0.0263

*Experiment 2*—Planted May 15, 1938; Harvest I, June 24; Harvest II, August 1.

*Experiment 3*—Planted August 12, 1938; Harvest I, September 8; Harvest II, October 24.

*Experiment 4*—Planted January 10, 1939; Harvest I, February 8; Harvest II, March 3; Harvest III, March 17; Harvest IV, April 8.

*Experiment 5*—Planted April 22, 1939; Harvest I, May 24; Harvest II, June 8; Harvest III, June 14; Harvest IV, June 27; Harvest V, July 5.

*Experiment 6*—Planted August 22, 1939; Harvest I, September 28; Harvest II, October 12; Harvest III, October 19; Harvest IV, November 2.

*Experiment 7*—Planted February 11, 1940; Harvest I, March 17; Harvest II, April 3; Harvest III, April 17; Harvest IV, May 6.

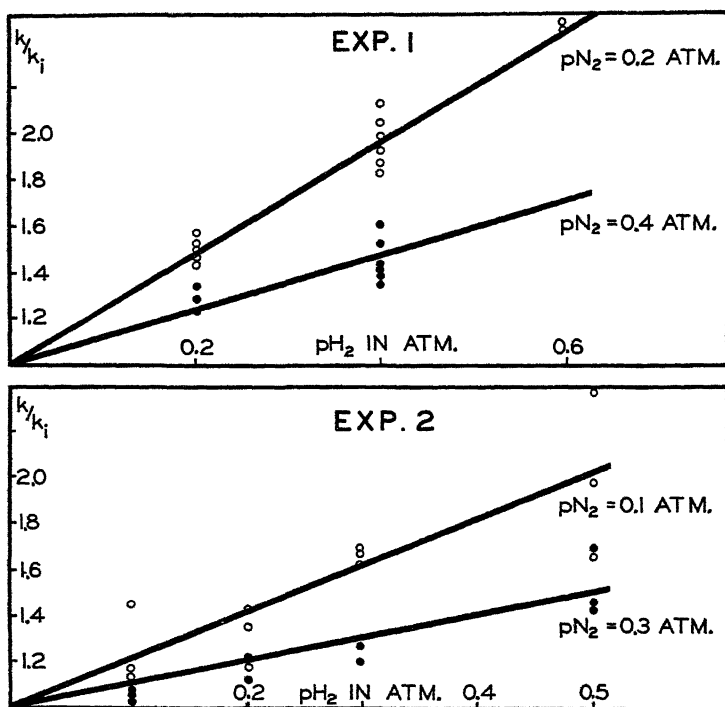


FIG. 1. Test for the type of inhibition by hydrogen of symbiotic nitrogen fixation in red clover. Based on Equation 6. Differences in the slopes of lines in Figs. 1, 2, and 4 indicate inhibition is competitive.

In all experiments the plants were initially placed under the gas mixtures at the first harvest. As Wilson (4) discussed the results of Experiments 5 and 6, only the summaries of these two will be given here.

## Results

Figs. 1 and 2 illustrate the data from Experiments 1 to 4 in which the  $pN_2$  was held constant and the  $pH_2$  varied. From an examination of Figs. 1 and 2 it appears probable that in each experiment the slopes of the two lines are distinctly different. Since the fit of the points to the lines is not exact, statistical analysis of the data is

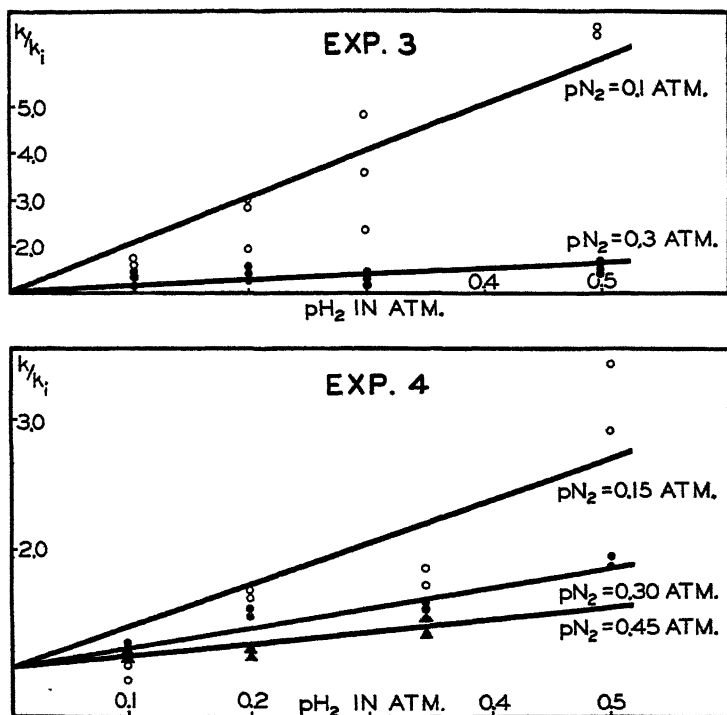


FIG. 2. Competitive inhibition of symbiotic nitrogen fixation in red clover by hydrogen.

desirable in order to demonstrate conclusively that the slopes actually differ. The cause of the deviation of the points from the "best" straight line is evident: the equations which dictate the method of plotting are based on a simple mechanism which assumes that the sole factors affecting the rate of fixation are the concentrations of substrate and inhibitor. Obviously, in long time experiments with plants this is not true, since other environmental conditions will influence the fixation reaction. In addi-

tion, in Experiments 2 and 3, a  $pN_2$  of 0.1 atmosphere was used in order that the rates of fixation in the absence of hydrogen would

TABLE II  
Summary of Statistical Tests on Slopes of Lines

Ex- peri- ment No.	$pN_2$	$N$	Line	Variance $s^2$	$t^*$	$t_{0.05}$
	<i>atmos- phere</i>					
1	0.2	14	$k/k_i = 1.0 + 2.390 pH_2$	0.0070	38.0	2.08
	0.4	9	$k/k_i = 1.0 + 1.106 pH_2$	0.00875		
2	0.1	12	$k/k_i = 1.0 + 1.960 pH_2$	0.03622	5.63	2.10-2.31
	0.3	10	$k/k_i = 1.0 + 0.920 pH_2$	0.0034		
3	0.1	11	$k/k_i = 1.0 + 9.531 pH_2$	0.7225	9.61	2.08-2.23
	0.3	12	$k/k_i = 1.0 + 0.933 pH_2$	0.01769		
4	0.15	8	$k/k_i = 1.0 + 3.499 pH_2$	0.1724	3.92	2.15
	0.30	8	$k/k_i = 1.0 + 1.777 pH_2$	0.0097		
	0.45	6	$k/k_i = 1.0 + 1.032 pH_2$	0.0059	6.52	2.18
5†	0.2	5	$k/k_i = 1.0 + 2.795 pH_2$	0.0425		
	0.4	6	$k/k_i = 1.0 + 0.922 pH_2$	0.0219	4.43	2.26
	<i><math>pH_2</math></i>					
6†	0.0	3	$1/k = 11.82 - 0.049 1/pN_2$	0.1011	6.77	4.30
	0.15	3	$1/k = 10.30 + 0.614 1/pN_2$	0.00203		
	0.35	3	$1/k = 10.68 + 0.889 1/pN_2$	0.0327	4.82	4.30
7	0.0	3	$1/k = 18.25 + 0.068 1/pN_2$	0.0465		
	0.15	3	$1/k = 19.09 + 0.514 1/pN_2$	0.1757	3.10	4.30
	0.35	3	$1/k = 18.41 + 1.300 1/pN_2$	0.0378		

\* If the  $t$  value exceeds  $t_{0.05}$ , the observed difference between the slopes of the lines (b) is significant. See foot-note 3.

† The details of Experiments 5 and 6 are described by Wilson (4).

differ. In this region the rate of fixation is a function of the  $pN_2$ ; hence the experimental error with this pressure was increased because of the difficulty in controlling the partial pressure of the



gases. At the other partial pressures of nitrogen the rate of fixation is independent of the  $pN_2$ , and therefore lack of exact control of this variable is without influence.

The fact that, unless the  $pN_2$  is rather low, the scatter of the points about the line is not appreciable suggests that, ordinarily, factors other than the  $pN_2$  and the  $pH_2$  play secondary rôles. When the ratio  $pH_2 : pN_2$  is high, the inhibition increases to a point at which an excessive carbohydrate-nitrogen balance obtains in the plant. This condition serves to accentuate the inhibition (4) with the result that the data become more erratic as two factors are definitely affecting the fixation reaction. A greater scatter of the points about the line is observed. The departure from a straight line, however, was in no case significant; the major disadvantage of this complicating factor is that, since the deviations of the points from the theoretical straight line are greater, a larger difference in the slopes must be obtained in order for it to be significant. Since both factors,  $pH_2$  and excessive carbohydrate-nitrogen balance, reduce the rate of fixation, the slope of the resulting line as well as its error (deviation of points from the line) is somewhat greater than would be the case if only the  $pH_2$  was effective. Thus the two sources of deviation from the theory tend to cancel one another.

Tests on the significance of the data are summarized in Table II. A measure of the closeness of fit of the points to their line is given by the value of  $s^2$ . In every case the value<sup>3</sup> of  $t$  greatly exceeded

<sup>3</sup> In the case of the simplified Equation 6, the estimate,  $b$ , of the true slope  $\beta$  is calculated from  $(\Sigma xy - \Sigma x)/\Sigma x^2$  in which  $x$  represents the concentration of inhibitor ( $pH_2$ ) and  $y$  the relative velocity of the reaction ( $k/k_i$ ). It is supposed that the  $x$  values are known without error and that for a given  $x$  the corresponding  $y$  is subject to an error with standard deviation,  $\sigma$ , independent of  $x$ . The estimate of  $\sigma^2$  provided by the deviations from regression is  $s^2 = [\Sigma(y - 1)^2 - b^2 \Sigma x^2]/(N - 1)$  where  $N$  is the number of paired observations. If  $b_1$  and  $b_2$  are two slopes and  $s_1^2$  and  $s_2^2$  the corresponding estimates of variance, the difference is tested with

$$t = \frac{b_1 - b_2}{\sqrt{\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2} [1/\Sigma x_1^2 + 1/\Sigma x_2^2]}}$$

with  $(N_1 + N_2 - 2)$  degrees of freedom. If  $s_1^2$  and  $s_2^2$  are not statistically homogeneous, the  $v$  test of Welch (5) is applied. In this test the "effective" degrees of freedom are known only to lie between  $N_1 - 1$  and  $N_1 + N_2 - 2$  ( $N_1 \leq N_2$ ); hence an exact value for  $t_{0.05}$  is not obtained, only its range. In

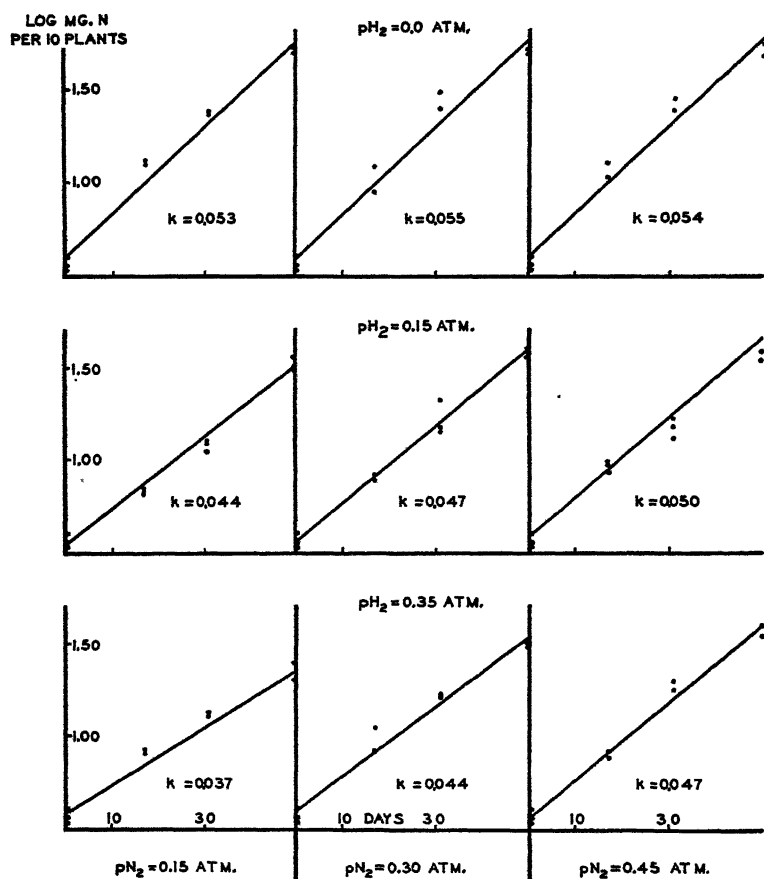


FIG. 3. Course of nitrogen fixation in red clover grown in atmospheres of different composition. Note that in the absence of hydrogen ( $p_{H_2} = 0.0$  atmosphere) the  $k$  values are independent of the  $p_{N_2}$  but not in its presence, indicating competitive inhibition.

testing for the difference between  $b$  values from the lines obtained when  $1/k$  is plotted against  $1/p_{N_2}$ , the  $t$  (or  $v$ ) test is applied with  $s^2 = [\Sigma(y - \bar{y})^2 - b^2 \Sigma(x - \bar{x})^2]/(N - 2)$ ;  $t$  has  $N_1 + N_2 - 4$  degrees of freedom. If  $N_1$  equals  $N_2$ , and  $\Sigma(x_1 - \bar{x}_1)^2 = \Sigma(x_2 - \bar{x}_2)^2$ , the  $t$  test can be used even though the variances are not homogeneous. The authors express their appreciation to Dr. Churchill Eisenhart, Station Statistician, for his aid in deriving the test for differences between  $b$  values from the simplified Equation 6 and for calling their attention to the necessity of using the  $v$  test when non-homogeneous variances are encountered.

that required to reach odds of 19:1 that the observed difference of the slopes was real. It is concluded, then, that the evidence from Experiments 1 to 4 supports the view that the inhibition is competitive.

Experiment 7 was one in which the  $pH_2$  was made the parameter and the  $pN_2$ , the variable; the analysis was based on Equation 1. In this experiment special care was taken to reduce experimental error, since the final lines were to be based on three points only,

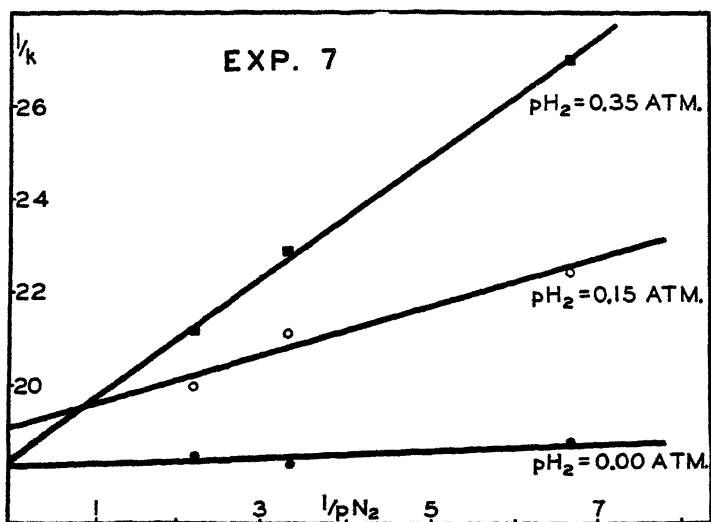


FIG. 4. Test for the type of inhibition of nitrogen fixation in red clover plants by hydrogen. Based on Equation 1.

so that very close fit was necessary in order to demonstrate that observed differences in the slopes were significant. Duplicate, and at times triplicate, samples were taken at each harvest. The course of the fixation is shown in Fig. 3 which illustrates that when  $\log mg. \text{ of } N$  was plotted against  $time$ , the fit of the points to the "best" straight line was quite satisfactory, so that the values of  $k$  based on these lines are reasonably free of error. As shown in Fig. 4, if the  $1/k$  values are plotted against the corresponding  $1/pN_2$ , the deviations from the best straight line are very slight. The statistical analysis given in Table II indicated that the dif-

ference between the slopes for the line corresponding to  $p\text{H}_2 = 0.0$  atmosphere and that corresponding to  $p\text{H}_2 = 0.15$  atmosphere did not quite reach the level of significance, primarily because each line contributes only one degree of freedom, so that a rather large value of  $t$  must be reached. On the basis of this single test one could only conclude that, although there appears to be a real difference, it was not statistically demonstrable. In a duplicate experiment (Experiment 6), this difference was clearly established. The slope of the line corresponding to a  $p\text{H}_2$  of 0.35 atmosphere was definitely greater than that for  $p\text{H}_2 = 0.15$  atmosphere as well as that for  $p\text{H}_2 = 0.0$  atmosphere (which was zero within experimental error). Tests also demonstrated that the observed differences between the intercepts of the lines were not statistically significant; *i.e.*, non-competitive inhibition was absent. In summary, the results from this experiment confirm those of the other four in that *competitive* inhibition of the fixation reaction by hydrogen is indicated.

#### SUMMARY

Two methods which determine the mode of action of an inhibitor for an enzyme reaction (whether *competitive* or *non-competitive*) are illustrated by experiments in which hydrogen acts as a specific inhibitor for symbiotic nitrogen fixation in red clover. The methods are based on plotting certain functions of the rates of reaction against (a) concentration of inhibitor for two or more concentrations of substrate, (b) reciprocal of concentration of substrate for two or more concentrations of the inhibitor. Straight lines are obtained whose slopes are tested for significant differences by appropriate statistical procedures.

Results from seven experiments, five of which are described in this paper, consistently favor the view that inhibition of the symbiotic nitrogen fixation reaction by hydrogen is *competitive*.

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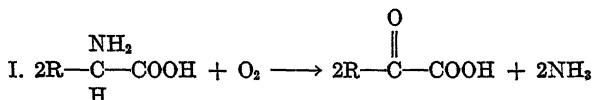
## SPECIFICITY OF THE *d*-AMINO ACID OXIDASE

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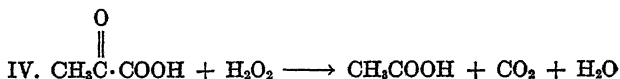
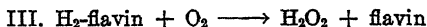
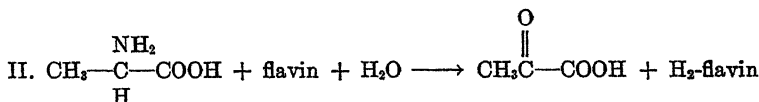
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Based on the determination of oxygen uptake, ammonia, and keto acids, the oxidation of *d*-amino acids by kidney and liver preparations can be represented by Equation I (1).



It has been shown, however, that hydrogen peroxide is formed during the oxidation (2, 3). Except under special conditions, the peroxide formed is rapidly decomposed by the catalase in the tissue preparations, and, therefore, does not affect the stoichiometrical relation indicated by Equation I.

The enzyme in kidney and liver catalyzing the oxidation of *d*-amino acids, *i.e.* the *d*-amino acid oxidase, is composed of a specific protein and flavin-adenine dinucleotide (4). The oxidation of *d*(-)-alanine by a reconstructed *d*-amino acid oxidase composed of a mixture of purified specific protein and flavin-adenine dinucleotide can be represented by Equations II, III, and IV (5).




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\* One of us (P. H.) is indebted to the John and Mary R. Markle Foundation for support during this work.

Flavin and  $H_2$ -flavin represent the oxidized and reduced forms of flavin-adenine dinucleotide respectively.

The preparations used in the most extensive studies of the oxidation of *d*-amino acids have been aqueous extracts of acetone-washed kidney (1, 6) or kidney extracts purified by adsorption on celite (2). In one case the aqueous extract was able to oxidize amines; *e.g.*, putrescine (6). The preparation purified by means of celite was able to oxidize *l*(-)-proline (2). Of the  $\alpha$ -amino acids tested in these studies only lysine,  $\alpha$ -aminoisobutyric acid (6), glycine, and  $\beta$ -hydroxyglutamic acid (1) were not oxidized. Karrer and Frank (7), however, report that the *d* form of histidine, dihydroxyphenylalanine, arginine, serine, aspartic acid, and glutamic acid is not oxidized by a reconstructed *d*-amino acid oxidase. They conclude that the oxidation of these amino acids observed by others was not due to the *d*-amino acid oxidase.

In the present work the oxidation of threonine, allothreonine, and pseudoleucine by an aqueous extract of acetone-washed kidney and by a reconstructed *d*-amino acid oxidase was studied. In addition the oxidation of a series of other amino acids by both preparations of the enzyme was tested.

#### EXPERIMENTAL

*Oxidation of d-Amino Acids by Aqueous Extract of Kidney*—Ground pig kidney was washed with acetone and dried *in vacuo* (4). The dried material was kept *in vacuo* over phosphorus pentoxide for 6 months before use. The extract was prepared by grinding 1 gm. of the dry material with sand and 10 ml. of water, and then centrifuging. 1 ml. of the supernatant fluid contained 14 mg. of solids of which 80 per cent was organic. The oxygen uptake of 1 ml. of this extract plus 1 ml. of 0.1 M pyrophosphate buffer, pH 8.3, was about 5 microliters per 300 minutes. The preparation did not oxidize any *l*-amino acid or putrescine.

The oxidation of the amino acids by the extract was tested by measuring oxygen uptakes in the usual Warburg apparatus. 1 ml. of the extract and 0.5 ml. of pyrophosphate buffer, 0.1 M, pH 8.3, were placed in the well of the vessel. The amino acid in 0.5 ml. of buffer was placed in the side arm. The insets contained alkali. The temperature was 37.5°. After the amino acid was added to the well, measurements were made until the oxygen uptake stopped. The data obtained are presented in

Table I. In those cases in which the *d* isomer was used the *dl* mixture was also studied. The results were the same.

The inability of the extract to oxidize glycine,  $\beta$ -alanine,  $\alpha$ -aminoisobutyric acid, and *dl*-lysine is in accord with the reports of others (1, 6). The failure of *dl*-pseudoleucine to be oxidized and the slow oxidation of *l*(+)-threonine<sup>1</sup> and levorotatory allo-threonine are compatible with the hypothesis that  $\beta$  substituents other than hydrogen inhibit or prevent the oxidation of *d*-amino acids by the *d*-amino acid oxidase (9, 10).

The results obtained with cystine and glutamic acid are not in accord with those of others. The low rate of oxidation of cystine is contrary to the experience of Krebs (1). In the case of glutamic acid, the low rate of oxygen uptake reported by Krebs is not unequivocal evidence for oxidation by the enzyme. However, the data for oxygen uptake and ammonia and ketonic acid production given by Felix and Zorn (6) and oxygen uptake and ammonia production given by Kögl, Herken, and Erxleben (11) seem conclusive. We are unable to explain the failure of the extract used in the present work to oxidize cystine and glutamic acid at an appreciable rate.

In the case of the amino acids *l*(+)-threonine and levorotatory allothreonine, which have not been studied previously, determinations of ammonia and  $\alpha$ -ketonic acids were made at the conclusion of the manometric measurements. For the ammonia determinations the material in the respirometer flask was acidified with trichloroacetic acid. The precipitate formed was removed by filtration. The ammonia was distilled from alkaline solution into dilute acid. The acid solution was nesslerized and the color obtained compared with that produced by suitable standards. The  $\alpha$ -ketonic acids were determined as described by Krebs (12). The determination is based on the fact that ceric sulfate in acid solution oxidizes 1 mole of  $\alpha$ -ketonic acid to 1 mole of carbon dioxide and 1 mole of an acid with 1 less carbon atom.

0.0042 mm of levorotatory allothreonine was oxidized with an

<sup>1</sup> Although the spacing of the groups on the  $\alpha$ -carbon of natural threonine is the same as in the case of other *l*-amino acids, *i.e.* natural amino acids, it has been designated *d*(-)-threonine by Meyer and Rose (8) because of its spatial similarity to *d*(-)-threose. Its isomer, although designated *l*(+)-threonine, is related with respect to the groups on the  $\alpha$ -carbon to the *d* form of other amino acids.



TABLE I  
*Oxidation of d-Amino Acids by Kidney Extract and Reconstructed d-Amino Acid Oxidase*

The amount of amino acid used is given in terms of microliters (1 mm = 22,400 microliters) of one isomer. One-half of the amount represents the theoretical oxygen uptake when the reaction proceeds according to Equation I. The full amount represents the theoretical oxygen uptake when the reaction proceeds according to Equations II to IV.

The data given are for a typical experiment.

	Extract			Reconstructed oxidase			
	Amount	O <sub>2</sub> uptake	Oxidation	O <sub>2</sub> uptake	Oxidation	CO <sub>2</sub> production	CO <sub>2</sub> production calculated on basis of O <sub>2</sub> uptake
	micro-liters	micro-liters	per cent	micro-liters	per cent	micro-liters	per cent
Glycine.....	148	0	0	0	0	0	0
d(-)-Alanine.....	126	59	94	118	94	110	93
β-Alanine.....	126	0	0	0	0	0	0
dl-Aminobutyric acid .....	108	54	100	95	88	95	100
α-Aminoisobutyric acid....	216	0	0	0	0	0	0
d(+)-Leucine.....	86	40	93	75	87	70	93
d(-)-Isoleucine.....	86	45	105	81	94	76	94
dl-Norleucine.....	86	39	91	80	93	70	88
dl-Pseudoleucine.....	86	0	0	0	0	0	0
d(-)-Valine.....	96	47	98	90	94	82	91
dl-Norvaline.....	96	42	88	89	93	83	93
dl-Serine.....	54	26	97	10	18	4	40
l(+)-Threonine*	84	10	24	8	10	3	37
l-Allothreonine† .....	84	35	83	15	18	8	53
dl-Cystine.....	46	1	4	0	0	0	0
dl-Methionine.....	76	37	97	68	90	65	96
dl-Aspartic acid.....	84	39	93	20	24	10	50
dl-Glutamic " .....	76	2	5	0	0	0	0
dl-Proline.....	86	42	98	76	89	0	0
dl-Phenylglycine.....	66	27	82	13	20	6	30
d(+)-Phenylalanine.....	68	29	85	59	87	55	93
dl-Tyrosine.....	62	33	106	37	60	20	54
dl-Tryptophane.....	54	26	96	12	22	5	42
dl-Arginine.....	54	10	37	10	19	4	40
dl-Lysine.....	62	0	0	0	0	0	0
d(+)-Histidine.....	62	10	32	10	16	4	40

\* See foot-note 1 in the text for an explanation of the sign defining the configuration.

† l indicates rotation, not configuration.

uptake of 0.0014 mm of oxygen and the production of 0.0020 mm of ammonia. Based on the reaction indicated by Equation I the ammonia production was 72 per cent of that expected from the oxygen uptake. In the case of 0.0042 mm of *l*(+)-threonine, the oxygen uptake was 0.0006 mm, the ammonia production 0.0008 mm. The ammonia production was 66 per cent of that expected from the oxygen uptake. For comparison ammonia determinations were also made in the case of the other amino acids which were slowly oxidized; *i.e.*, *dl*-serine, *dl*-aspartic acid, *dl*-arginine, and *d*(+)-histidine. The ammonia production was 60 to 80 per cent of that expected from the oxygen uptakes.

In the oxidation of 0.0042 mm of levorotatory allothreonine 0.0015 mm of oxygen was used. The addition of ceric sulfate after the measurements of oxygen uptake produced 0.0027 mm of carbon dioxide. This indicates that the production of  $\alpha$ -ketonic acid was 90 per cent of that expected from the oxygen uptake. In the oxidation of 0.0042 mm of *l*(+)-threonine 0.0005 mm of oxygen was used. 0.0004 mm of carbon dioxide was produced after the addition of ceric sulfate. This indicates that the production of  $\alpha$ -ketonic acid was 40 per cent of that expected from the oxygen uptake.

The data obtained indicate that the oxidation of levorotatory allothreonine by the *d*-amino acid oxidase follows the same pattern as that of other *d*-amino acids; *i.e.*, the utilization of 1 atom of oxygen and the production of 1 mole of ammonia and  $\alpha$ -ketonic acid per mole of amino acid. The data in the case of *l*(+)-threonine suggest that the oxidation follows the same pathway.

As has been demonstrated by others, no ammonia was produced in the oxidation of *d*(+)-proline (2, 12). Krebs (12) identified the product of the oxidation of *d*(+)-proline as  $\alpha$ -keto-*d*-aminovaleric acid. With the procedures described by Krebs it was possible in the present work to demonstrate the formation of an  $\alpha$ -ketonic acid as the product of the reaction and to isolate it as the 2,4-dinitrophenylhydrazone, m.p. 224°. The hydrazone had the properties described by Krebs.

*Oxidation of d-Amino Acids by a Reconstructed d-Amino Acid Oxidase*—A preparation of the specific protein component of the oxidase was prepared by the method of Warburg and Christian (4) from an extract of the dried pig kidney used above. Each

mg. of the preparation contained about 0.15 mg. of protein. The flavin-adenine dinucleotide was prepared from yeast as the barium salt (4). The purity was 0.73, estimated spectrophotometrically.

The oxidation of the amino acids by the reconstructed system was tested by measuring oxygen uptakes and carbon dioxide production manometrically. The amino acid was added to a mixture of 3 mg. of protein preparation and 2.4  $\gamma$  of dinucleotide in a total volume of 2 ml. of 0.05 M pyrophosphate buffer, pH 8.3. The temperature was 37.5°. Measurements were made until the oxygen uptake stopped. No O<sub>2</sub> uptake was obtained with the mixture without added substrate in 135 minutes. The concentration of flavin was such that doubling the concentration increased the rate of oxidation 5 per cent. The activity of this preparation decreased about 90 per cent in 135 minutes, when shaken in air at 37.5°. Bernheim (13) has shown that the *d*-amino acid oxidase activity of tissue preparations decreases when shaken in air, but does not decrease in nitrogen. The decrease is due to oxidation of the protein, since the activity of the reconstructed system can be restored by the addition of more protein, but not by the addition of flavin.

The data in Table I show that the amino acids oxidized by the kidney extract are also oxidized by the reconstructed system. With the amino acids that were completely oxidized the oxygen uptakes were in agreement with those predicted from a summation of Equations II to IV; *i.e.*, 1 mole of oxygen per mole of *d*-amino acid. The carbon dioxide production, except in the case of *dl*-proline, was likewise that predicted from the equations. In the case of the amino acids that were slowly oxidized the carbon dioxide production was considerably less than the amount expected from the oxygen uptake. The measurement of carbon dioxide production was less exact than that of the oxygen uptake owing to the large blank, which was of the order of 25 microliters of carbon dioxide.

Contrary to Karrer and Frank (7) we find that the *d* form of histidine, aspartic acid, arginine, and serine is oxidized by the reconstructed oxidase. The rate of oxidation is low and the extent of oxidation is small. The low rate of oxidation and the lability of solutions of the purified protein when exposed to oxygen may account for the inability of these workers to demonstrate oxidation of the amino acids in question.

In the case of *dl*-proline no carbon dioxide was produced, al-

though the oxygen uptake was 1 mole of oxygen per mole of *d*-amino acid. When the product of the oxidation of 0.0038 mm of *dl*-proline was oxidized with ceric sulfate, 0.0035 mm of carbon dioxide was obtained. The product of the oxidation of the same amount of *dl*-proline yielded 0.0016 mm of amino nitrogen. In a large scale experiment with 20 mg. of *dl*-proline, the hydrazone of  $\alpha$ -keto-*d*-aminovaleric acid was obtained. Apparently the *d* isomer of proline is oxidized to the  $\alpha$ -ketonic acid. The peroxide that is formed preferentially oxidizes the *l* isomer of proline, rather than the ketonic acid, to a product yielding carbon dioxide on ceric sulfate oxidation, but containing no amino nitrogen.

#### SUMMARY

The oxidation of a series of amino acids by a kidney extract and a reconstructed *d*-amino acid oxidase was tested.

The *d* form of alanine,  $\alpha$ -aminobutyric acid, leucine, isoleucine, norleucine, valine, norvaline, serine, methionine, aspartic acid, proline, phenylglycine, phenylalanine, tyrosine, arginine, and histidine, *l*(+)-threonine, and levorotatory allothreonine were oxidized by both preparations.

Since levorotatory allothreonine is oxidized by the *d*-amino acid oxidase, it is probably related structurally to the *d* forms of the other amino acids.

Glycine,  $\alpha$ -aminoisobutyric acid, pseudoleucine, cystine, glutamic acid, and lysine were not oxidized by either preparation of the oxidase.

We are indebted to Dr. R. C. Corley for a sample of pseudoleucine and to Dr. H. E. Carter for samples of *d*(+)-, *l*(-)-, and *dl*-threonine, and *d*-, *l*-, and *dl*-allothreonine.

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# A MICROBIOLOGICAL ASSAY TECHNIQUE FOR PANTOTHENIC ACID WITH THE USE OF *PROTEUS MORGANII*\*

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Several methods for the assay of pantothenic acid have been reported in the literature. They have been based on the growth response of yeast (1), bacteria (2, 3), and chicks (4, 5) to the vitamin. The relative merits of these methods have been discussed in an earlier paper (3).

In a previous publication (6) it was shown that pantothenic acid is one of the growth factors required by *Proteus morganii*. Subsequent experiments in which synthetic crystalline calcium pantothenate (Merck) was employed in a chemically defined medium indicated that the organism responded to exceedingly low concentrations of this substance; raising the calcium pantothenate level within limits resulted in a corresponding increase in growth. On the basis of this reaction to pantothenate a technique has been developed for its assay. Since the method possesses certain desirable features, its application to routine assays of pantothenic acid in natural materials may be warranted.

## EXPERIMENTAL

*Test Organism*—A strain of *Proteus morganii* designated as Strain 21 was used in this study. The stock culture was carried on nutrient agar slants and transferred at 2 week intervals.

*Preparation of Inoculum*—A trace of growth from a 24 hour agar slant culture was taken up on the end of a wire transfer needle and

\* We wish to thank Merck and Company, Inc., for generously supplying the synthetic calcium pantothenate and the  $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\alpha$ -butyrolactone employed in this study.

inoculated into 10 ml. of the assay medium (described below) containing 0.0005  $\gamma$  per ml. of crystalline calcium pantothenate. After 24 hours incubation, 1 drop (approximately 0.05 ml.) of this first subculture, delivered from a 1.0 ml. pipette, was placed in 10 ml. of similar medium. Following a 24 hour incubation period the growth of this second subculture was comparable to the turbidity of a No. 1 McFarland nephelometer tube and it was used to inoculate the assay medium in test determinations. 1 drop of this culture, delivered from a 1.0 ml. pipette, was always used to inoculate tubes containing 10 ml. of media.

Inoculation of the assay medium (lacking pantothenate) gave consistently negative results; growth could not be detected by any of the methods employed.

*Basal Medium*—Previous investigations in this laboratory have shown that *Proteus morganii* can be cultivated in a chemically defined medium of the following composition:  $\text{KH}_2\text{PO}_4$  4.5 gm.,  $(\text{NH}_4)_2\text{SO}_4$  0.5 gm.,  $(\text{NH}_4)\text{Cl}$  0.5 gm.

The reaction was adjusted to pH 7.4 and the mixture was tubed in 9.5 ml. quantities and autoclaved. Prior to inoculation the medium in each tube was supplemented with  $\text{Fe}(\text{SO}_4)(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$  (0.002 M in 0.02 M HCl) 0.20 ml.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.4 per cent in  $\text{H}_2\text{O}$ ) 0.10 ml., glucose (50 per cent solution) 0.10 ml., nicotinic acid ( $10^{-3}$  M in  $\text{H}_2\text{O}$ ) 0.10 ml., cystine (M/1500 in 0.1 M HCl) 0.10 ml.; calcium pantothenate (aqueous solution), 0.01  $\gamma$ , contained in 0.10 ml.

*Assay Medium*—Although the organism grew well on serial subculture in the basal chemically defined medium described, it was apparent that the mass of growth, as well as the rate of growth, did not approach the level which was obtained with glucose-meat infusion broth, or other similar bacteriological media.

Attempts were made to increase the growth of *Proteus morganii* by supplementing the basal medium with numerous substances known to serve as growth accessory or stimulating factors for bacteria. None of the media prepared, however, supported growth equal to glucose-meat infusion.

Naturally, it is pertinent that a medium for the assay of a substance should be adequate in all respects except for the substance to be assayed. Since we were unable to produce such a substrate by supplementing a simple chemically defined medium with

numerous growth factor substances of known identity, it was necessary to resort to more complex materials.

10 per cent solutions of casein, yeast extract, liver, and proteose-peptone were prepared in  $N$  NaOH and autoclaved at 15 pounds pressure for 1 hour to effect complete inactivation of pantothenic acid. Each solution was then neutralized with glacial acetic acid, filtered to remove the precipitate, and finally autoclaved at 15 pounds pressure for 10 minutes. These served as stock solutions from which additions were made to the basal chemically defined medium. After several preliminary experiments in which the basal medium was supplemented with various quantities of the above solutions, it was found that if 0.1 ml. of the alkali-treated proteose-peptone solution was added per 10 ml. of basal media a substrate was obtained which supported growth equal to that obtainable in glucose-meat infusion broth. Not only was growth markedly increased by the addition of 0.1 per cent alkali-treated proteose-peptone to the medium, but the test organism could now initiate growth at a level of 0.0002  $\gamma$  of calcium pantothenate per ml. rather than at 0.005  $\gamma$ , as was the case when the chemically defined basal medium was employed.

Consequently, the medium selected for use in assaying pantothenic acid was the basal medium described above in which the calcium pantothenate was omitted, and to which the alkali-treated proteose-peptone was added to give a final concentration of 0.1 per cent (0.1 ml. of stock solution added to 10 ml. of media). This will be referred to as the *assay medium*. The assay medium was prepared in liter quantities and then dispensed in 10 ml. amounts into sterile cotton-stoppered test-tubes.

### Results

*Response of Proteus morganii to Added Pantothenate in an Otherwise Pantothenic Acid-Free Medium*—Repeated experiments conclusively demonstrated that inoculation of the basal medium without pantothenate or the assay medium gave no detectable growth. However, the addition of calcium pantothenate in small amounts permitted moderate growth to take place in these media.

The response of *Proteus morganii* to various concentration levels of calcium pantothenate was determined by three methods: (1) by measuring the increase in turbidity of the medium after a 24



hour incubation period with the aid of a Klett-Summerson photometer; (2) by determining the amount of bacterial nitrogen in 50 ml. of media with a micro-Kjeldahl technique; (3) by measuring the pH of the substrate after 24 hours incubation with a Leeds and Northrup universal potentiometer.

The results obtained by these methods are presented in Table I, in which are also included corresponding determinations ob-

TABLE I  
*Response of Proteus morganii to Calcium Pantothenate in Pantothenic Acid-Free Media*

Amount of Ca pantothenate added	Response of test organism as determined by					
	Turbidity		pH		Bacterial N	
	Chemically defined medium	Assay medium	Chemically defined medium	Assay medium	Chemically defined medium	Assay medium
$\gamma$ per ml.						
0 (Uninoculated control)	0	0	7.4	7.4	0.0	0.0
0	0- 0*	0- 0*	7.4	7.4	0.0	0.0
0.0001	1- 1	7- 7	7.4	7.4	0.0	0.0
0.0005	6- 6	60- 60	7.4	7.4	0.0	0.259
0.001	14- 14	74- 72	7.4	7.4	0.0	0.576
0.005	36- 36	103-104	7.4	7.35	0.175	0.788
0.01	45- 47	118-118	7.08	6.95	0.420	0.924
0.05	107-105	152-152			0.806	1.044
0.1	121-123	169-169	5.93	5.85	1.151	1.458
0.2	123-124	172-175	5.67	5.57	1.319	1.850

\* Duplicate determinations.

The turbidity was measured on the Klett-Summerson photometer.

The values for bacterial N are in mg. per 50 ml.

tained with the basal chemically defined medium. These data show that growth is absent in the pantothenate-free media. However, when as little as 0.0002  $\gamma$  of calcium pantothenate was added per ml. of the assay medium, a growth resulted which was demonstrable macroscopically and measurable by the turbidimetric method. Growth at this same level could not be detected by either pH or bacterial nitrogen determinations; consequently, turbidity readings were chosen for assay purposes in subsequent experiments. In the chemically defined basal medium the first

detectable growth occurred when 0.005  $\gamma$  of pantothenate was added per ml.

A standard curve for use in assaying materials was obtained by determining the relationship between growth and the concentra-

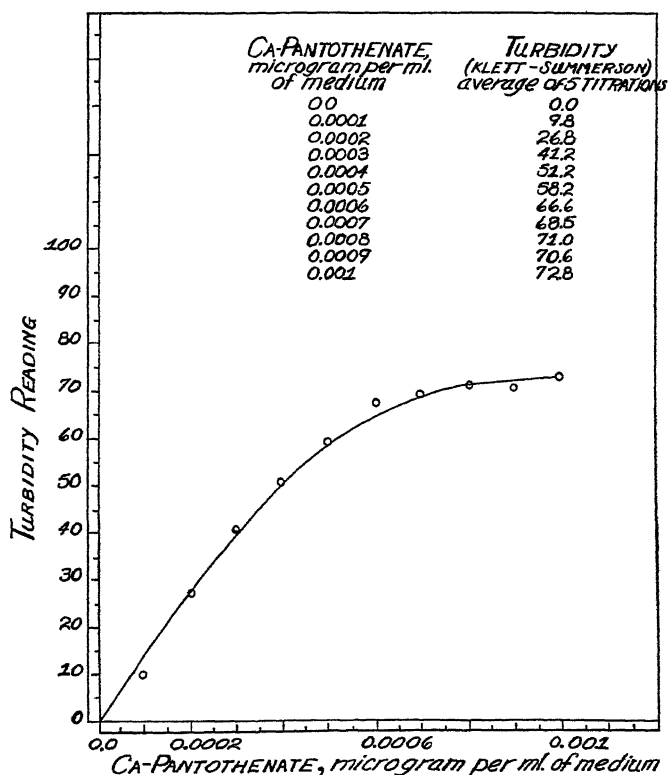


FIG. 1. Relationship between the turbidity and the concentration of calcium pantothenate in a chemically defined medium plus 0.1 per cent alkali-treated peptone.

tion of calcium pantothenate ranging from 0.0001 to 0.001  $\gamma$  per ml. of the assay medium. Values for this relationship are tabulated and plotted in Fig. 1. The curve lends itself very well for the evaluation of the pantothenic acid content of extracts from various materials. It is necessary, however, to establish such a curve each time a group of assays is made, since it has been ob-

served that slight deviations in the curve result on different days.

*Application of Method for Assay of Extracts of Natural Materials*—Aqueous extracts of natural materials were prepared as previously described (3, 7). The material was autoclaved for 30 minutes at 15 pounds pressure, after which any voluminous precipitate was removed by filtration through paper. The relatively clear filtrates were then autoclaved for 10 minutes at 15 pounds pressure and the sterile solutions served as the stock material for assay.

A preliminary assay of each extract was first carried out by a titration technique to obtain the approximate pantothenate concentration in the specimen. This was done as follows: 10-fold dilutions were prepared from each extract and 0.1 ml. of each dilution was incorporated in 10 ml. of the assay medium; the tubes were inoculated and after 24 hours incubation were observed macroscopically for evidence of growth. In previous experiments with synthetic crystalline pantothenate, it was observed that 0.0002  $\gamma$  of calcium pantothenate per ml. of assay medium afforded growth of the test organism which was evident by macroscopic observation. Half of this amount, or 0.0001  $\gamma$  per ml., resulted in no growth detectable macroscopically. Consequently, in the preliminary assay of a substance if the tube containing 0.1 ml. of a 1:100 dilution showed growth but 0.1 ml. of the 1:1000 dilution was negative, it would indicate that 0.1 ml. of the lower dilution contained at least 0.002  $\gamma$ , but less than 0.02  $\gamma$ , of pantothenate. Furthermore, it follows that the 0.1 ml. of the 1:1000 dilution contained less than 0.002  $\gamma$  of pantothenate.

To obtain more exact information two or three amounts of either of these two dilutions were selected which by interpretation of the above results should give turbidity readings between 20 and 60 on the standard curve. These tubes were then inoculated, incubated for 24 hours, the turbidity read, and the pantothenate concentration calculated from the curve.

In most instances no interference has been encountered by the opacity or color of the extracts, since the dilutions used have been sufficient to eliminate this error. However, when the amount of extract does impart either opacity or color to the assay medium, it is necessary to clarify the extract, previous to making an assay, by employing an adsorbent such as kieselguhr.

TABLE II  
*Assay of Pantothenate from Extracts of Natural Substances*

Material assayed	Amount of extract incorporated in 10 ml. assay media	Klett-Sumner-son nephelometer reading	Pantothenic acid equivalent	Pantothenate concentration per mg. substance
			$\gamma$	$\gamma$
Bacto-peptone	0.4 ml., 1:100 dilution	20-20*	0.0016-0.0016	0.04 -0.04
	0.8 " 1:100 "	40-40	0.003 -0.003	0.0375-0.0375
Bacto-liver	0.1 " 1:100 "	39-37	0.0029-0.0028	0.29 -0.28
Bacto-yeast	0.2 " 1:1000 "	34-34	0.0026-0.0026	1.30 -1.30
extract	0.3 " 1:1000 "	51-51	0.0038-0.0038	1.296 -1.296
Casein	0.1 " 1:10 "	25-25	0.0019-0.0019	0.019 -0.019
	0.2 " 1:10 "	51-51	0.0038-0.0038	0.019 -0.019

\* Duplicate determinations.

TABLE III  
*Recovery of Added Calcium Pantothenate from Several Substrates*

Specimen to which Ca pantothenate was added	Amount of pantothenate in specimens as determined by previous assay	Amount of crystalline Ca pantothenate added	Total calculated pantothenate content	Amount recovered by assay	Per cent recovery
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	
Bacto-peptone	0.0016	0.002	0.0036	0.0032	88
	0.0030	0.002	0.0050	0.0048	96
Bacto-liver	0.0029	0.002	0.0049	0.0053	108
Bacto-yeast extract	0.0026	0.002	0.0046	0.0050	108
	0.0038	0.002	0.0058	0.0062	106
Casein	0.0018	0.002	0.0038	0.0041	107
	0.0040	0.002	0.0060	0.0061	101
Alkali-treated specimens					
Bacto-peptone	0	0.002	0.002	0.0019	95
		0.003	0.003	0.0031	103
Bacto-liver	0	0.002	0.002	0.0018	90
		0.003	0.003	0.0028	93
Bacto-yeast extract	0	0.002	0.002	0.0021	105
		0.003	0.003	0.0032	106
Casein	0	0.002	0.002	0.0019	95
		0.003	0.003	0.0026	87

*Quantitative Recovery of Pantothenate from Various Substrates*—Experiments were carried out patterned after those previously employed (3) for the quantitative recovery of pantothenic acid.

The natural pantothenate content of Bacto-peptone, Bacto-yeast extract, Bacto-liver, and casein was first determined. Aqueous extracts of each were prepared and the assay was conducted as described. Results of these assays, which are presented in Table II, show a close agreement between values obtained with duplicate samples, as well as with those obtained with different amounts of the same sample.

Following this, a known amount of calcium pantothenate was added to a sample of each of the above extracts and the resulting mixtures were then assayed. Similar assays were made in which calcium pantothenate was added to alkali-treated samples of the above extracts. The results are presented in Table III, and the agreement obtained between the assay values and the actual pantothenate content in all instances provides evidence for the consistency in duplication of results and for the specificity of the test.

Additional evidence for the specificity of this assay procedure is that it has been impossible to replace the pantothenic acid with any one or a combination of known bacterial growth factors. Furthermore, the intact pantothenic acid molecule is required, since in experiments in which the pantothenate molecule was replaced by its two components ( $\beta$ -alanine and  $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\alpha$ -butyrolactone) growth failed to occur.

#### DISCUSSION

In this assay procedure a test organism is employed which is capable of responding to a lower dosage of pantothenate than the organisms used in other methods described in the literature. When as little as 0.0002  $\gamma$  of synthetic calcium pantothenate is added per ml. of the assay medium, visible growth occurs. This makes it possible to determine the pantothenate content of extremely small amounts of natural substances, a fact which may be significant, since other non-specific factors which might affect the test, such as inhibitory substances and interference by color or opacity, can be avoided by dilution.

Preparation of inoculum is extremely simple and there is little

chance for contamination to occur. The composition of the assay medium approaches chemical definition and consequently lends itself to accurate duplication.

#### SUMMARY

A microbiological technique, in which *Proteus morganii* is employed, has been described for assaying the pantothenic acid content of natural materials. Evidence has been presented which indicates that the response of the test organism to pantothenate is highly sensitive and specific. The organism requires only about 0.0002  $\gamma$  of calcium pantothenate per ml. of medium to initiate visible growth. Results obtained with this test indicate that recovery of pantothenate is practically quantitative.

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# ON THE PREPARATION OF ADENOSINE TRIPHOSPHATE

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WITH THE TECHNICAL ASSISTANCE OF KRIKOR SERAIDARIAN

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Since efforts to isolate adenosine triphosphate from brain by the various procedures described in the literature (Fiske and Subbarow, 1929, b; Lohmann, 1931, 1932; Barrenscheen and Filz, 1932) led to poor yields or to impure products, we attempted first to revise the method of isolation. The procedure described below has given satisfactory yields of pure salts of adenosine triphosphate.

In the method described by Fiske and Subbarow (1929, b) the compound was prepared from the neutralized trichloroacetic acid extract of muscle by precipitation with mercuric acetate in the presence of 2 per cent acetic acid, followed by removal of the mercury as sulfide and finally by precipitation with calcium chloride and alcohol from HCl solution. For the sake of further purification the entire procedure was repeated. Barrenscheen and Filz (1932) adopted this general procedure in preference to the more complicated one of Lohmann (1931). Although they stated their yield as silver salt to be 30 to 38 per cent from rabbit muscle and as high as 64 per cent for dog muscle,<sup>1</sup> we were unable to secure more than a 15 per cent yield by their procedure, and therefore

<sup>1</sup> The highest content we have noted for dog muscle excised rapidly and frozen in liquid air is 59 mg. of adenosine triphosphate phosphorus per 100 gm., and the average for five experiments was 49 mg., calculated on the assumption that the hydrolyzable phosphorus (7 minutes hydrolysis in N HCl at 100°) represents two-thirds of the adenosine triphosphate phosphorus. In the experiment cited by Barrenscheen and Filz 13 gm. of the silver salt were reported to have been obtained from 3 kilos of dog muscle, which corresponds to 48.8 mg. of P per 100 mg. If the yield were 64 per cent, the muscle should have had an original content of 76 mg. of adenosine triphosphate P per 100 gm., a figure much higher than we have observed.



examined the various steps to determine the reasons for loss. In the first step (addition of alkaline calcium chloride<sup>2</sup>) only 60 to 75 per cent of the adenosine triphosphate is precipitated, as judged by determining both the easily hydrolyzed phosphorus and the purine nucleotide (Kerr, 1940). The results were not significantly different when the quantity of calcium chloride added was 0.25 volume (Fiske and Subbarow, 1929) or 0.1 volume (Barrenscheen and Filz, 1932).

In the second operation, after solution of the precipitate in 2 per cent acetic acid, mercuric acetate precipitates somewhat less than 90 per cent of the nucleotide present. If mercuric acetate is added directly to the neutralized trichloroacetic acid filtrate in the presence of 2 per cent acetic acid as recommended by Fiske and Subbarow (1929, b), the loss of nucleotide is over 25 per cent.

On precipitation of the silver salt from  $\text{HNO}_3$  solution with 25 per cent alcohol as recommended by Barrenscheen and Filz (1932), approximately half of the material may be lost.

These losses may be reduced considerably by taking advantage of the fact that mercuric acetate in the presence of 0.1 to 0.2 per cent acetic acid quantitatively precipitates the purine nucleotides from tissue filtrates (Kerr, 1940), leaving inorganic phosphate in solution.<sup>3</sup>

We prefer to prepare the compound as a barium rather than a calcium salt, because the lower solubility of the former permits repeated precipitation from aqueous solution with relatively little loss, and also because of the ease with which the cation may be removed when the silver or sodium salts are to be prepared. The separation of adenosine diphosphate, adenylic acid, inosinic acid, the pyridine nucleotides, and other nitrogenous impurities is accomplished by several precipitations of the barium salt from

<sup>2</sup> Barrenscheen and Filz (1932) apparently understood from the publication of Fiske and Subbarow (1929, b) that precipitation by alkaline calcium chloride formed the first step in this procedure. Since 25 to 40 per cent of the material is lost in this one operation, we believe that this interpretation was not intended.

<sup>3</sup> It is remarkable that although mercuric acetate precipitates the major portion of inorganic phosphate from pure solutions containing trichloroacetate and 0.2 per cent acetic acid, it precipitates practically none of the inorganic phosphorus contained in or added to trichloroacetic acid extracts of muscle or brain.

aqueous solution (Lohmann, 1931, 1932; Warburg and Christian, 1936).

*Preparation of Barium Salts*—Muscle, excised rapidly from an anesthetized animal, is ground in a chilled meat chopper and received in a weighed vessel containing 20 per cent iced trichloroacetic acid. After being weighed again, the mixture is diluted to make the proportion of muscle to acid 1:5 and the concentration of acid 8 to 10 per cent, then allowed to stand on ice with occasional mixing for half an hour. The protein precipitate is separated and the filtrate<sup>4</sup> neutralized to phenolphthalein with 40 per cent NaOH.

Glacial acetic acid is added to a concentration of 0.2 per cent, followed by 5 cc. of 20 per cent mercuric acetate solution<sup>5</sup> per 100 cc. of filtrate, and the mixture is allowed to stand until the precipitate has settled. This is separated by centrifugation, washed once with 0.5 per cent mercuric acetate, and then suspended in water (volume in cc. about one-fifth the number of gm. of muscle used).<sup>6</sup> The suspension is cooled in ice and treated with H<sub>2</sub>S for an hour, with occasional shaking. The mercuric sulfide is separated by centrifuging, again suspended in water, and treated a second time (15 minutes) with H<sub>2</sub>S. The combined supernatant fluids contain all of the nucleotide and hydrolyzable phosphorus found in the original protein-free filtrate, and only traces of inorganic phosphorus.

In order to remove iron the iced solution is made slightly alkaline with N NaOH and the volume of alkali noted.<sup>7</sup> About 10 gm. of sodium acetate are added to insure flocculation of the ferrous sulfide, and H<sub>2</sub>S is again passed in for 10 minutes. The ferrous sulfide is filtered off, the cold solution is again acidified with a volume of 3 N HCl equivalent to the NaOH used, and H<sub>2</sub>S is removed by aeration. The barium salt is at once precipitated

<sup>4</sup> Before neutralization a small portion should be set aside for determination of organic hydrolyzable phosphorus (Lohmann, 1928) if information about yield is desired.

<sup>5</sup> The mercuric acetate is dissolved in 2 per cent acetic acid.

<sup>6</sup> Unless the procedure can now be carried through to the precipitation of the barium salt by alcohol without delay, it is essential that the decomposition with H<sub>2</sub>S should be postponed until the next day.

<sup>7</sup> Adenosine triphosphate is slowly converted into adenylic acid and pyrophosphate in alkaline solution even on ice; hence the period during which the solution remains alkaline must be as brief as possible.

by addition of 25 per cent barium acetate solution (25 cc. per kilo of muscle) and 2 volumes of alcohol. As soon as the precipitate has flocculated, it may be centrifuged, dissolved in 0.1 N HCl,<sup>8</sup> and reprecipitated with alcohol. These two precipitations remove the major part of a nitrogenous impurity and the last trace of inorganic phosphorus. Losses of adenosine triphosphate up to this point are less than 5 per cent. The proportion of hydrolyzable to total phosphorus is about 66 per cent, but the atomic ratio of N:P is about 5.4:3.

To separate the remaining impurities, the barium-alcohol precipitate is centrifuged,<sup>9</sup> dissolved in HCl as before (volume in cc. about one-tenth the number of gm. of muscle used), and precipitated by addition of 25 per cent barium acetate solution (15 cc. per kilo of muscle sample) together with sufficient Ba(OH)<sub>2</sub> to make the solution barely alkaline to phenolphthalein.<sup>10</sup> The precipitate is dissolved and reprecipitated in the same way until the atomic ratio of total P to total N is 3:5. Two such precipitations followed by two with barium acetate alone (barium hydroxide being omitted) usually suffice.

The dibarium salt thus prepared is washed twice with water and once with 50 per cent alcohol to remove excess barium acetate, twice with 95 per cent alcohol and once with ether to remove water, and then powdered and air-dried. For complete dehydration it should be dried in a vacuum over P<sub>2</sub>O<sub>5</sub> at 100°. The yield is approximately 60 per cent of the adenosine triphosphate in the original filtrate. On addition of 2 volumes of alcohol to a solu-

<sup>8</sup> In this and all succeeding solutions of the barium precipitates the most efficient method is first to add water, stopper the centrifuge bottle, and shake to suspend the precipitate. On addition of sufficient 3 N HCl to make the entire solution approximately 0.1 N, most of the precipitate dissolves. When large preparations (800 gm. of muscle or more) are handled, two extractions may be required, with persistent shaking. The extracts in all cases are filtered to separate insoluble material. On account of the lower solubility of the compound when cooled in ice it is preferable to work rapidly at room temperature.

<sup>9</sup> The acid barium salt suspended in alcohol is relatively stable and may be left overnight but the succeeding steps must be carried through without delay if decomposition is to be avoided.

<sup>10</sup> From the discarded supernatant liquids, alcohol precipitates material in which the hydrolyzable P represents about 40 per cent of the total, and the atomic ratio of P:N is 2:5.8.

tion of the neutral barium salt in 0.1 N HCl a mixture of mono- and dibarium salts is precipitated. A second precipitation from HCl solution yields the monobarium salt. Since this is soluble in water, it should be washed only with alcohol and ether. It may be completely dehydrated in a vacuum over  $P_2O_5$  at  $100^\circ$ .

The corresponding calcium salts may be prepared by similar procedures. After the removal of iron two precipitations by alcohol (4 volumes) and two with calcium chloride saturated with  $Ca(OH)_2$  yield a product with the correct ratio of P:N but a low yield (38 per cent). After two precipitations by alcohol from HCl solution a pure monocalcium salt is obtained.

*Analytical Methods*—Carbon and hydrogen were determined by the micromethods of Pregl (Pregl and Roth, 1935). All of the nucleotide preparations after drying in a vacuum over  $P_2O_5$  absorb moisture very rapidly on exposure to air; hence it was necessary to dry and weigh all samples for analysis in the special weighing bottle and vacuum drying pistol designed for this purpose (Pregl and Roth, 1935, p. 68). On combustion of the nucleotides some charred material usually remains encased in the fused residue of phosphate, but ignition with vanadium pentoxide results in complete oxidation.<sup>11</sup> We have found it preferable to burn the sample in the usual way, then to withdraw the boat momentarily for addition of a piece of recently fused  $V_2O_5$ , and reignite, rather than to add the  $V_2O_5$  before the combustion, with consequent absorption of moisture from the air during the delay.

Nitrogen was determined by a micro-Kjeldahl procedure (Kerr, 1940), and total phosphorus colorimetrically (Fiske and Subbarow, 1925). Barium was precipitated as sulfate from hot 0.1 N HCl solution, transferred by suction to a porous bottomed crucible (Pregl and Roth, 1935, p. 146), and weighed after three ignitions with intermediate washings. Silver was determined by titration with 0.01 N thiocyanate after ashing with sulfuric acid (Hitchings, 1933), the results by this procedure agreeing closely with those obtained by weighing the silver as chloride.

*Preparations*—The compound precipitated by barium acetate from 0.1 N HCl solution and dried over  $P_2O_5$  at  $100^\circ$  in a vacuum

<sup>11</sup> This is the procedure used in the micro laboratory of the chemical department of the Kaiser Wilhelm Institute at Heidelberg, according to a personal communication from Dr. Wallenfels to Dr. George Fawaz.

## 126 Preparation of Adenosine Triphosphate

(Cenco Hyvac oil pump) had a composition agreeing with that calculated for the anhydrous dibarium salt.

*Analysis*— $C_{10}H_{12}O_{13}N_5P_3Ba_2$  (777.88)

Calculated. C 15.43, H 1.56, N 9.00, P 11.97, Ba 35.3

Found. " 15.38, " 1.95, " 8.95, " 11.79, " 33.5

Lohmann's (1931) analysis of his air-dried salt, precipitated at "neutral reaction," corresponded to that of a dibarium salt with 6 molecules of water, 5.61 of which were given up on drying in a high vacuum over  $P_2O_5$  at  $78^\circ$ . Our preparation after drying in air for over 2 weeks gave up water corresponding to 13.87 per cent of the anhydrous residue (equivalent to 5.95 molecules) on being dried in a vacuum over  $P_2O_5$  at  $100^\circ$  for 3 hours. Barrenscheen and Filz (1932) stated that their compound after drying in the desiccator retained 6 molecules of water, only 4 of which were lost at  $100^\circ$  over  $P_2O_5$  in a vacuum.

The compound precipitated twice by alcohol from the solution of the barium salt in 0.1 N HCl and dried in a vacuum over  $P_2O_5$  for 1 hour at  $100^\circ$  had a composition agreeing with that calculated for the anhydrous monobarium salt.

*Analysis*— $C_{10}H_{14}O_{13}N_5P_3Ba$  (642.52)

Calculated. N 10.91, P 14.50, Ba 21.40

Found. " 10.93, " 14.69, " 20.1

*Silver Salts*—Fiske and Subbarow (1929, b) stated that the calcium salt may be converted to the silver salt ( $C_{10}H_{13}O_{13}N_5P_3Ag_3$ ) by precipitation with  $AgNO_3$  from  $HNO_3$  solution. Barrenscheen and Filz (1932) precipitated the silver salt from 0.83 N  $HNO_3$  by addition of alcohol to 25 or 30 per cent concentration, then redissolved and reprecipitated it in order to obtain material pure enough for analysis. They reported no silver determinations. We find that the compound precipitated by  $AgNO_3$  and alcohol from a solution of the calcium salt containing  $HNO_3$  (either 0.1 or 0.5 N) contains usually only 2 atoms of silver and 0.5 of an equivalent of calcium. The silver salt prepared without the use of alcohol also contained calcium. Iron, if not previously removed, will also be found in the silver salt replacing part of the silver.

The pure trisilver salt was prepared from free adenosinetriphosphoric acid (made from the barium salt by addition of the

calculated equivalent of  $\text{H}_2\text{SO}_4$ )<sup>12</sup> by precipitation with silver nitrate solution in the presence of 0.1 N  $\text{HNO}_3$  and 0.5 volume of alcohol. The precipitate was washed several times with alcohol and finally with ether and was then dehydrated in a vacuum for 1 hour over  $\text{P}_2\text{O}_5$  at  $100^\circ$ . The composition agreed with that calculated for the anhydrous trisilver salt.

*Analysis*— $\text{C}_{10}\text{H}_{13}\text{O}_{13}\text{N}_5\text{P}_3\text{Ag}_3$  (827.87)

Calculated. C 14.49, H 1.58, N 8.46, P 11.24, Ag 39.1

Found. " 14.78, " 2.06, " 8.52, " 11.16, " 39.5

A tetrasilver salt was obtained by adding free adenosine-triphosphoric acid (prepared by addition of the calculated amount of  $\text{H}_2\text{SO}_4$  to the barium salt<sup>12</sup>) to an excess of 1 per cent silver acetate solution. Addition of 0.5 volume of alcohol caused additional precipitation. After being washed with alcohol and ether the product was dehydrated over  $\text{P}_2\text{O}_5$  at  $100^\circ$  in a vacuum. At  $100^\circ$  in N HCl 67.1 per cent of the phosphorus was hydrolyzed in 15 minutes. The compound was white, but on long exposure to light the exterior became grayish, and the inner portions yellow. The trisilver salt assumes a reddish violet color on exposure to light.

*Analysis*— $\text{C}_{10}\text{H}_{12}\text{O}_{13}\text{N}_5\text{P}_3\text{Ag}_4$  (934.75)

Calculated. C 12.84, H 1.30, N 7.49, P 9.94, Ag 46.2

Found. " 12.91, " 2.18, " 7.41, " 9.49, " 46.8

Barrenscheen and Filz (1932) described a compound the composition of which agreed with that calculated for the tetrasilver salt of adenosine triphosphate, although no silver determination was reported. Wagner-Jauregg (1936) repeated their procedure but found the preparation to consist of a mixture of inosine di- and triphosphate. The compound prepared by Barrenscheen and Filz was stated to form an acid solution. Our preparation, after removal of silver by means of 1 per cent NaCl solution, yielded a solution neutral to phenolphthalein.

<sup>12</sup> The amount of  $\text{H}_2\text{SO}_4$  required is calculated from the total P content of the preparation in HCl solution. The compound is again precipitated by barium acetate and washed to remove excess barium. The slight excess of  $\text{H}_2\text{SO}_4$  resulting from this calculation (due to the loss of material during reprecipitation and washing) is unobjectionable.

## 128 Preparation of Adenosine Triphosphate

Both the tri- and the tetrasilver salts dissolve readily in 1 per cent NaCl solution forming opalescent transparent solutions from which AgCl separates slowly on standing at room temperature, rapidly at 100°.

A soluble mercury preparation, possibly a double salt of sodium and mercury, was also prepared. On addition of mercuric acetate to the sodium salt of pure adenosinetriphosphoric acid (prepared by neutralizing the free acid with NaOH) no precipitate forms,<sup>13</sup> but addition of 1.5 volumes of alcohol causes the separation of a precipitate containing both mercury and sodium, easily soluble in water. On evaporation of the aqueous solution to dryness in a vacuum at room temperature a clear glassy mass with conchoidal fracture results. For analysis the material precipitated by alcohol was washed with alcohol and ether and dried in a vacuum at 100° over P<sub>2</sub>O<sub>5</sub>. Addition of water acidified with HCl resulted in an opalescent solution from which a small amount of mercurous chloride separated on heating. This was filtered off and weighed. Mercuric mercury was precipitated by H<sub>2</sub>S, filtered through a porous bottomed crucible, washed with CS<sub>2</sub>, and weighed as HgS. Phosphorus, nitrogen, and sodium were determined in the filtrate. For sodium estimation an aliquot portion was evaporated in a quartz dish, digested with HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>, ignited, and then dissolved in 25 cc. of water. Phosphorus was removed by shaking with 0.2 gm. of Ca(OH)<sub>2</sub>. From this point the procedure recommended by Butler and Tuthill (1931) for solutions with minimal sodium content was continued, the treatment with ammonium perchlorate being omitted. The data obtained by analysis of the mercury-sodium complex are given in Table I. The entire method was tested on known mixtures of sodium salts and pure adenosine-triphosphoric acid and found to be exact.

Whether the preparation consists of a double salt or of a mixture, it is obvious that the mercury and sodium cannot all be attached to the phosphorus if the formula of Lohmann (1935)

<sup>13</sup> Although mercuric acetate forms no precipitate when added to a solution of the pure sodium salt, it nevertheless precipitates nucleotides quantitatively from the neutralized trichloroacetic acid extract of tissues. In such solutions the adenosine triphosphate must be present as the sodium salt, but possibly is attached also to other groups.

with 4 replaceable hydrogen atoms is to be accepted. Possibly 1 atom of mercuric mercury may be attached to the free amino group of adenine.

Since the proportion of total phosphorus hydrolyzed in the presence of  $N$  HCl at  $100^\circ$  is occasionally used as a means of estimating adenosine triphosphate, it is of interest to compare our findings with those of Lohmann (1931) and Fiske (1934). The former stated that exactly two-thirds of the organic phosphorus was converted to inorganic in 7 minutes, whereas Fiske found two-thirds split in 15 minutes. For twenty-two different preparations of the dibarium salt, each with a P:N ratio close to the

TABLE I  
*Analysis of Mercury-Sodium Complex*

	<i>per cent</i>	<i>atoms</i>
Hg (ous).....	1.62	0.08
" (ic).....	42.7	2.13 (4.26 H <sup>+</sup> equivalents)
Na.....	3.58	1.56
P.....	9.15	2.95
N.....	7.00	5.00

The sum of the cations (H<sup>+</sup> equivalents) is 5.90 atoms; the ratio of cations to P, 5.90:2.95 or 2:1.

theoretical, we find the amount hydrolyzed within 15 minutes to average  $67.9 \pm 1.4$  per cent of the total. For six preparations of the acid salt the average was  $67.2 \pm 1.4$ , and for five silver salts  $65.1 \pm 1.0$ .

#### SUMMARY

Procedures are described for the preparation of the barium and silver salts of adenosine triphosphate, including a tetrasilver salt not previously described.

A soluble complex mercury preparation is described.

The author is indebted to his colleague, Dr. George H. Fawaz, for valuable advice, particularly in the use of the micromethods of Pregl.



## 130      Preparation of Adenosine Triphosphate

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## NOTES ON THE PREPARATION OF MUSCLE ADENYLIC ACID

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Although a number of methods have been published for the preparation of adenylic acid by hydrolysis of adenosine triphosphate (Lohmann, 1931, 1932; Barrenscheen and Lang, 1932), we encountered sufficient difficulty in securing a pure product to justify presenting details of a method which finally yielded good results. In this method use is made of mercuric acetate for concentrating the adenylic acid from dilute solution. Various details of other published procedures are utilized, and the conditions are defined for securing the best yield of an analytically pure product.

In order to permit repeated recrystallization of the final product, a minimum of 2.5 mM of adenosine triphosphate (2 gm. of the anhydrous dibarium salt<sup>1</sup>) should be used for the preparation. This is brought into solution by shaking with water (40 to 50 cc. per mM) and HCl, a portion is removed for determination of total phosphorus,<sup>2</sup> and the number of mM in the entire batch is calculated (total phosphorus divided by 93). The barium salt is then reprecipitated by neutralizing with Ba(OH)<sub>2</sub> solution, with phenolphthalein as indicator. The suspension is immersed in a boiling water bath and the calculated amount of Ba(OH)<sub>2</sub> required for the hydrolysis (the equivalent of 2 cc. of N Ba(OH)<sub>2</sub> per mM of adenosine triphosphate) is added from a burette during a period of 20 minutes, additions being made at intervals of a

<sup>1</sup> A method for the preparation of this salt is described in the preceding paper (Kerr, 1941).

<sup>2</sup> Measure 1 cc. into a 10 cc. graduated cylinder, add a few drops of 10 N H<sub>2</sub>SO<sub>4</sub>, and dilute to 10 cc. Filter off the BaSO<sub>4</sub> and use 1 cc. of the filtrate for determination of total phosphorus (Fiske and Subbarow, 1925).

minute. The heating is continued for another 10 minutes (30 minutes in all). Although the separation of adenylic acid from the pyrophosphate split off during the alkaline hydrolysis is based on the solubility of barium adenylate, practically none of the latter is found in the supernatant fluid. Hence the precipitate after cooling is brought into solution by means of HCl, diluted to a volume of 700 cc. per mm, and then reprecipitated by addition of  $\text{Ba}(\text{OH})_2$  until phenolphthalein is just reddened. The precipitated barium pyrophosphate is allowed to settle and the supernatant fluid containing the adenylate is separated by siphoning and centrifuging. The solution is made 0.2 per cent acid with acetic acid, and 0.05 volume of 20 per cent mercuric acetate is added. After settling overnight the precipitated mercury adenylate is separated by decanting and centrifuging, washed once with 0.5 per cent mercuric acetate solution, and then suspended in  $\text{H}_2\text{O}$  (a volume of 20 cc. per mm of original material). A few drops of 2 N  $\text{H}_2\text{SO}_4$  are added to insure removal of traces of barium carried down with the mercury precipitate.  $\text{H}_2\text{S}$  is then passed through the suspension for an hour with occasional shaking,  $\text{HgS}$  is removed by centrifuging and filtering, and the filtrate is aerated. Together with the washings the volume of solution should not exceed 25 cc. per mm. Analysis of this solution for total phosphorus should show a concentration of about 1 mg. per cc., losses of about 30 per cent having occurred up to this point. The hydrolyzable phosphorus (15 minutes hydrolysis in N HCl at  $100^\circ$ ) is, however, about 18 per cent of the total, indicating the presence of some di- or triphosphate of adenosine, since only 2 per cent of adenylic acid is hydrolyzed under these conditions. The polyphosphates may be partially separated by fractional precipitation with acetone.

Acetone is added gradually to the solution at room temperature until the first permanent turbidity is produced, but not over 1.5 volumes, and the flask is placed on ice overnight. The amorphous precipitate which forms on addition of 1.5 volumes of acetone contains about 7 per cent of the phosphorus present. The ratio of phosphorus to nitrogen in the precipitate is 1.2:5, and the hydrolyzable phosphorus is about 24 per cent of the total. This precipitate is discarded.

To the supernatant fluid acetone is again added to the point of

turbidity. On standing overnight on ice adenylic acid separates in crystalline form. Acetone is added repeatedly in quantities sufficient to cause turbidity until a second 1.5 volumes (total, 3 volumes) have been added. Under these conditions 72 per cent of the adenylic acid will crystallize out. Another 17 per cent of the phosphorus can be precipitated by addition of acetone up to 5 volumes, but this fraction also is amorphous and has a P:N ratio similar to that of the first impure fraction.

The crystalline adenylic acid is by no means pure, having a P:N ratio of 1.07:5 and a low melting point. The crystals are freed from acetone by gentle heat, then dissolved from the walls of the flask in a minimum of boiling water, and filtered into a pointed centrifuge tube. With washings the volume should not exceed 4 cc. per mm of original material, and the solution should contain from 5 to 8 mg. of phosphorus per cc. When cooled on ice with occasional stirring, crystalline adenylic acid separates. This is centrifuged while still cold and the supernatant fluid separated.<sup>3</sup> Six to eight recrystallizations from hot water are required to complete the purification. Although the ratio of phosphorus to nitrogen may be correct after five crystallizations, the melting point will be found to be as low as 182°, whereas further recrystallization yields material with a melting point of 189–190°. The crystals are finally washed with alcohol and ether. Anhydrous material is obtained from the air-dried salt on exposure to a high vacuum over P<sub>2</sub>O<sub>5</sub> at room temperature (28°), no further loss occurring at 78°.

*Analysis*—C<sub>10</sub>H<sub>14</sub>N<sub>5</sub>O<sub>7</sub>P (347.27)

Calculated. C 34.56, H 4.06, N 20.17, P 8.93

Found. " 34.93, " 4.75, " 19.95, " 8.98

On rapid heating (1° in 10 seconds) the crystals darkened at 186° and melted with decomposition and effervescence at 189° (uncorrected). The procedure recommended by Mulliken (1905) was followed.

Embden and Zimmermann (1927) observed a melting point of 194°, Lohmann (1931) reported 197–198°, and Barrenscheen and

<sup>3</sup> If the centrifuging is done while the tube is still cold, the loss of adenylic acid in the mother liquor will be 6.2 mg. (0.55 mg. of P) per cc. The mother liquor may be reserved for further recovery of adenylic acid of lower purity.

## 134 Preparation of Muscle Adenylic Acid

Lang (1932) 193°. Jachimowicz (1937) reported melting points of 198° for the preparation of Embden, 196° for adenylic acid prepared by means of phosphatase, and 196° for synthetic adenylic acid.

On hydrolysis in *N* HCl at 100°, 2.01 per cent of the phosphorus was converted to inorganic phosphorus in 15 minutes, 4.79 per cent in 30 minutes, and 10.98 per cent in 60 minutes. Since adenine is completely liberated under these conditions within 15 minutes or less (Lohmann, 1931; Levene and Tipson, 1937), the values reported for 30 and 60 minutes actually represent the rate of decomposition of ribose phosphate.

### SUMMARY

Details of a method for the preparation of adenylic acid by alkaline hydrolysis of the barium salt of adenosine triphosphate are presented.

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# THYROIDAL ACTIVITY OF IODINATED SERUM ALBUMIN

## IV. THE EFFECT OF PROGRESSIVE IODINATION\*

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The relief of myxedema with iodoproteins of extrathyroidal origin and the preparation of metabolically active iodoproteins have been described in a previous communication (1). The proteins used had an iodine content of about 15 per cent. The amount of iodoprotein needed to give a standard response<sup>1</sup> according to Lerman and Salter (1) contained about 170 mg. of iodine and corresponded to approximately 1 gm. of original protein.

It was obvious that by far the largest part of the iodine was present in some form that is completely inactive in the relief of myxedema. Therefore, for the study of the chemical reactions underlying the formation of the physiologically active material, it seemed desirable to determine at which iodine concentration the first sign of activity manifested itself and at which concentration the activity became maximal.

*Preparation*—The protein used was horse serum albumin obtained from the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health. The globulins had been precipitated from the serum with alcohol without further precipitation of the albumin.

To 1 liter of albumin in 25 per cent alcohol, usually containing from 45 to 50 gm. of protein, were added 240 cc. of concentrated

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<sup>1</sup> A "standard response" is the response observed on administration of thyroxine polypeptide containing 0.5 mg. of iodine.

ammonia and varying amounts of 1 N iodine in potassium iodide (approximately 25 per cent potassium iodide). During this process the iodine was added in 10 cc. portions to the protein solution; each successive portion was added only when all free iodine of the lot previously added had disappeared. The reaction mixture was allowed to stand for 15 minutes after the reaction was completed. It was then neutralized with aqueous hydrochloric acid (1 volume of concentrated acid to 1 volume of water), with simultaneous addition of ice to keep it at room temperature. The reaction of the mixture was adjusted to provide maximal precipitation; *i.e.*, at about pH 4.5 to 5.0. The precipitate was left to settle until the next day. It was then washed several times with water and dissolved with sodium hydroxide at pH 8 to 9. The solution was analyzed for total nitrogen, ammonia nitrogen, and total iodine. The iodine percentage was calculated as the ratio

$$\frac{\text{Iodine} \times 100}{\text{Protein N} \times 6.25 + \text{iodine}}$$

In some cases when the inorganic material had not been completely washed out, a small correction for inorganic iodine still present was made, as follows: The residual iodide was estimated from the amount of ammonia nitrogen in the solution by assuming that the ratio of ammonium chloride and total iodide still present had not changed as a result of washing.

*Progressive Iodination of Albumin*—Table I shows the results of the iodination of two different samples of horse serum albumin. The amount of iodine that enters into the protein molecule increases as more iodine is added, up to a certain maximum content. In this series, the maximum iodine bound per 100 gm. of iodo-protein amounts to 15 per cent (Table I, last column). If more iodine is added, not only does no more iodine enter the protein, but the final product usually has a lower iodine content (Table I, 1940, Protein 3). The amount of iodine added is in most cases only slightly more than twice the amount bound, a finding which suggests that the reaction is primarily a substitution and that not much oxidation takes place. The preparations made in the spring of 1940 all have a lower iodine content, for the same amount of iodine added, than those in the autumn of 1939. This is probably

due to a difference in the sera. Both series show essentially a linear relation between iodine added and iodine bound up to 45 gm. of free iodine supplied per 100 gm. of protein.

Although the evidence is still incomplete, judging from the observations of earlier investigators (2) iodine will first enter into

TABLE I  
*Iodination of Horse Serum Albumin*

Protein No.	Albumin used	Added iodine	Iodopro- tein obtained	Added I per 100 gm. P	Bound I per 100 gm. P	I added I bound	Per cent I in iodo- protein
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		
1939 series							
Ia	162	15.1	122	9.32	4.03	2.33	3.9
IIa	243	30.2	224	12.4	5.83	2.13	5.5
IIIa	243	45.3	231	18.6	8.69	2.14	8.0
IVa	227	60.4	241	26.6	12.2	2.18	10.9
Va	247	96.0	266	38.8	17.1	2.27	14.6
IIIb	246	45.3	243	18.3	8.47	2.16	7.8
IIIc	240	45.3	238	18.8	8.73	2.15	8.03
IIb	47.6	6.04	46.4	12.6	5.95	2.12	5.61
IVb	47.6	12.7	48.4	26.7	10.6	2.52	9.58
1940 series							
1	48.4	7.75	44.8	16.0	7.02	2.28	6.6
2		10.4	47.4	21.5	8.75	2.46	8.2
3		25.4	48.9	52.5	13.2	3.97	11.8
4		19.0	52.0	39.4	16.4	2.40	14.4
5		20.3	52.9	42.0	14.9	2.81	13.2
6		21.6	54.4	44.6	17.6	2.53	15.3
7		8.25	34.3	17.1	7.7	2.22	7.2
8		11.4	46.1	23.6	9.4	2.51	8.7
9		12.7	41.1	25.3	10.6	2.38	9.7
10	58.6	14.6	48.0	24.9	9.8	2.77	9.0
11		16.5	58.5	28.2	11.1	2.54	10.0
12	293	101	295	34.5	12.4	2.78	11.1
13	140	17.4	123	12.5	5.6	2.23	5.3

the tyrosine of the protein molecule. It has been stated also that when all the tyrosine has been transformed into diiodotyrosine eventually histidine and possibly other cyclic amino acids become iodinated. If the tyrosine content of serum albumin is assumed to be 4.65 per cent (3), the iodine content should be 6.10 per cent when all the tyrosine has been iodinated and no other constituent



of the protein molecule has taken up iodine. This amount is less than one-half the percentage of iodine we have found in optimally iodinated albumin. Subsequently, if histidine also is iodinated completely, the total iodine content theoretically should be increased to 10.8 per cent, if one accepts the value of 3.4 per cent cited by Cohn (3). Obviously, even this assumption would not account for all the iodine actually found combined. Even lower values would follow from the value of 1.2 per cent given by Schmidt (4).

### *Method of Assay*

The procedure followed in estimating the activity of these preparations was that of Salter, Lerman, and Means (5). The details of the assay have been discussed in an earlier publication (6). Accordingly, only a brief synopsis will be given here.

*Qualitative Assay*—The relief of human myxedema or cretinism produces striking and highly specific changes in physical appearance, mental activity, heat and water balance. All of the positive assays reported here showed unmistakable qualitative evidence of "thyroidal" activity.

*Quantitative Assay*—For this purpose three main criteria were used.

1. *Rate of Recovery*—Salter, Lerman, and Means (5) studied the reaction of the completely myxedematous adult to thyroxine or its polypeptide. They found that the daily administration of 0.5 mg. of iodine *in this form* produced a rise in basal metabolic rate which was nearly linear with time for 14 consecutive days. During this fortnight the basal metabolic rate characteristically rose from  $-40$  to  $-5$ . This response was termed the "standard response." The most accurate assays included in this report are those which approximately reproduced the "standard" response. In assays of thyroxine polypeptide in which four patients were employed, the accuracy had been estimated at better than  $\pm 15$  per cent; simultaneous assays in the hands of Professor Reid Hunt had been no better than  $\pm 50$  per cent by his acetonitrile test in mice.

2. *Level of Recovery*—Somewhat less accurate estimates of potency can be made by comparing the *level of recovery* with other levels produced by known thyroid medication. This method was employed by Lerman and Salter (7) to assay various therapeutic

agents. The basis for it has been described by Means and Lerman (8). In brief, the permanent level of metabolism reached by an athyreotic adult is a function of the daily supply of thyroid hormone. For example, if the basal metabolic rate is  $-40$  with *no* daily medication, it will approximate  $-25$  with 0.1 mg. of thyroxine iodine daily,  $-15$  with 0.2 mg.,  $-10$  with 0.4 mg., or  $-5$  with 0.5 mg. after only 2 weeks of continuous medication. Similarly, 1.0 mg. daily would give a basal metabolic rate of  $+10$  to  $+15$ . Slightly higher levels result from longer administration of the drug. These values have been established by Thompson, McLellan, Thompson, and Dickie (9), by Boothby and Plummer (10), and by Means and Lerman (8). The last authors point out that the duration of medication must be taken into consideration.

This second criterion is most satisfactory when the effect of successive medications is compared in the same patient, the one medication serving to calibrate the metabolic standard for the other.

*3. Blood Cholesterol*—The concentration of blood cholesterol is notoriously variable when random samples are taken from an unselected population. Nevertheless, it has been shown by Gilligan, Volk, Davis, and Blumgart (11) and by Man, Gildea, and Peters (12) that in a single individual, well controlled (as in a hospital), the level of blood cholesterol varies inversely with the basal metabolic rate in a characteristic fashion. Therefore, once the individual is calibrated, comparative assays may be gaged by this concentration.

With these several criteria in mind, and with due experience in observing myxedematous patients, it is possible not merely to say that a given preparation has thyroidal activity, but also approximately what percentage of the "standard dose" is reproduced by an unknown preparation. Because in work previously published (1) it was found that slightly more than 1 gm. of serum protein, optimally iodinated, produced the "standard response" of Salter, Lerman, and Means (5), all assays in this report were conducted on the basis of 168 mg. of protein nitrogen as the unit of daily dosage. With this unit of protein varying amounts of iodine were combined, and the resultant potency studied. Detailed data are given in Table II.

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The medication was always given dissolved in a large excess of water, buffered with sodium bicarbonate. It was administered

TABLE II  
*Iodine Content Versus Thyroidal Activity*

Per cent iodine	Nitrogen dose	Basal metabolic rate		Cholesterol, mg. per cent		Time fed	Estimated activity per 100 mg. N
		Before feeding	After feeding	Before feeding	After feeding		
	mg.						
3.9	168						0
5.3	555	-40	-49	362	346	June 29-July 9	0
6.6	605	-3	-8			May 14-23	25
7.2	470	-1.7	-3			" 3-14	45
8.0	168	-20	-5			Oct. 5-15	40
	210	W -18; H -10	W -12; H -5	280	117	Sept. 29-Oct. 13	35
8.2	336	-24	-24			Apr. 4-14	15
	168	-24	-20			" 4-12	25
	336	-20	-20			" 12-20	
8.7	168	-45	-26			" 27-May 9	
	336	-26	-19			May 9-20	40
9.7	168	+4	-4	170	168	" 4-18	100
	168	-7.7	-1.7	224	Lost	Apr. 20-May 3	120
10.9	336	+4	+16	223	190	Mar. 1-15	75
	168	-36	-18	372	206	" 4-15	80
	168	-18	-14	206	208	" 15-21	
	168	W +10; H +18	W +16; H +18	107	95	" 7-Apr. 12	
11.8	168	+9	-8	200	224	Apr. 5-20	100
13.2	168	-24	+5			" 15-28	150
14.4	168	W +16; H +18	W +14; H +12	95	56	" 12-May 22	100
14.6	168	" -27; " -22	" +18; " +24	213	61	Jan. 11-Feb. 13	100
	168	" +18; " +24	" +10; " +19	61	107	Feb. 13-Mar. 7	
	168	-24	-14	300	284	Jan. 12-26	
	336	-14	+2	284	238	" 26-Feb. 9	90
	336	+2	+7	238	184	Feb. 9-23	
	168	-40	-20			" 21-Mar. 6	
15.3	168	-20	+4	206	170	Apr. 20-May 4	140

W, calculated on the basis of weight; H, calculated on the basis of height.

"on an empty stomach," usually early in the morning. In this way maximal absorption was insured.

*Biological Response*

In Fig. 1 the metabolic response has been plotted against the percentage of iodine in the iodoprotein. The results fall conveniently into three categories: preparations with less than 6 per cent iodine show no activity; preparations with between 6 and 9 per cent iodine show, in relation to the "standard dose" containing 168 mg. of nitrogen,<sup>2</sup> 15 to 45 per cent of the standard response;

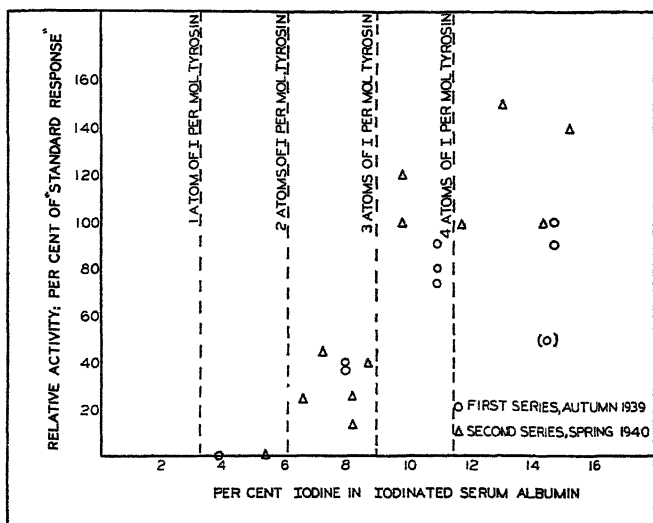


Fig. 1. Iodine content of horse serum albumin and its thyroïdal activity

preparations with more than 9 per cent iodine show a response of 80 to 150 per cent of the standard.

The spread in the response to different preparations of approximately the same iodine content is such that it cannot be decided definitely whether further increase of the iodine content above 10 per cent increases the physiological activity. The general conclusion that full activity is reached at an iodine content of from 9 to 10 per cent seems justifiable, however. Incidentally, it will be observed that a single preparation with an iodine content of 14.6

<sup>2</sup> 168 mg. of nitrogen was found by Lerman and Salter (1) in optimally iodinated proteins to produce, on the average, a "standard response."

per cent showed in one test an activity of only 50 per cent of the standard, but the same preparation tested on two other patients showed 90 and 100 per cent, respectively.

It is often assumed that the physiological activity of thyroglobulin is determined by the thyroxine content. Of known compounds only thyroxine and to a much smaller degree diiodothyronine have been shown to have sufficient metabolic activity to be of significance in mammalian or clinical physiology. It is therefore natural to assume that the physiological activity of the serum protein iodinated *in vitro* is due to thyroxine, diiodothyronine, or perhaps some close derivative. Ludwig and von Mutzenbecher (13) have been able to isolate thyroxine from casein iodinated *in vitro*, and their finding has been verified by Harington and Rivers (14). Since thyronine has never been isolated from proteins, diiodotyrosine presents itself as the most likely precursor of thyroxine. As Harington and Rivers have pointed out (14), the formation of thyroxine from diiodotyrosine is difficult to reconcile with our concepts of the chemistry of those compounds. On the other hand, von Mutzenbecher has reported (15) that thyroxine is formed from diiodotyrosine incubated in 0.1 N sodium hydroxide at 37° for 14 days, and this finding has been confirmed by Block (16) and by Curlin.<sup>3</sup> Diiodotyrosine, as such, when fed even in very large doses has little or no effect on patients suffering from myxedema (17).

As mentioned already, the first sign of activity in our iodoalbumin manifests itself at an iodine content slightly above 6 per cent. This corresponds to full iodination of the tyrosine to diiodotyrosine, assuming that there is not simultaneous iodination of other amino acids capable of binding iodine. The next step leading to the formation of the active principle could then be explained by several possible mechanisms. (1) Further addition of iodine would give rise to a side reaction between 2 molecules of diiodotyrosine to form thyroxine or some other thyronine derivative. (2) Thyronine might already be present in the protein molecule, and part of the iodine added beyond 6 per cent might enter the thyronine

<sup>3</sup> Curlin, L. C., private communication; unpublished data in Ph.D. thesis, University of Chicago (1939).

nucleus. (3) Some other unknown active material might be formed.

We have not been able thus far to isolate thyroxine from any of our active products. Since our most active materials would need to contain only from 0.5 to 0.7 mg. of thyroxine iodine per 150 mg. of total iodine, or about 0.5 per cent of the total iodine as thyroxine iodine, in order to give the observed metabolic response, this failure may well be due to the experimental difficulty of isolating such a small quantity. For the large amounts of material used in these metabolic experiments it was not feasible to employ crystalline albumin. Bonot (18), however, has reported the preparation of crystalline iodoalbumin from crystalline serum albumin (horse). It is interesting that his crystalline preparation contained, per 100 gm. of dry substance, 13.7 gm. of nitrogen and approximately 11.3 gm. of iodine. These figures are very close to the composition of our crude iodoalbumin at maximal endocrinological potency. One of us (W. T. S.) is engaged in preparing from crystalline serum albumin by Bonot's method a series of similar crystalline proteins at various stages of iodination. Of course the serum albumin fraction is a complex one, and it may be interesting ultimately to test various subfractions in order to ascertain whether these behave differently toward iodine.

#### SUMMARY

When serum albumin was iodinated in stages, thyroidal activity appeared at 6 per cent iodine and increased up to 10 per cent iodine content. The results obtained suggest (1) that diiodotyrosine must be formed before metabolic activity appears; and (2) that at least one additional iodine atom must be added to the protein molecule in order to produce a completely active iodoprotein.

Further addition of iodine to the albumin molecule apparently did not enhance the metabolic activity of the preparation.

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## THE EFFECT OF THIAMINE DEFICIENCY IN RATS ON THE EXCRETION OF PYRUVIC ACID AND BISULFITE-BINDING SUBSTANCES IN THE URINE\*

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Thiamine deficiency in all species thus far studied affects the metabolism of pyruvic acid. The lack, until quite recently (1), of a rapid specific method for estimating pyruvic acid necessitated the use of the rapid but less specific technique introduced by Clift and Cook (2). Occasionally it has been estimated by isolation as the 2,4-dinitrophenylhydrazone, by the method of Case (3) or a modification (4) of that method.

The Clift and Cook procedure (2) is based on the fact that pyruvic acid (as well as other ketones and aldehydes) combines with bisulfite. Thompson and Johnson (4) were the first to attempt to correlate pyruvic acid with the great increase in bisulfite-binding substances in the blood of thiamine-deficient rats and pigeons. Quantitative measurements in the polyneuritic pigeon of pyruvic acid as the 2,4-dinitrophenylhydrazone demonstrated that the increase in bisulfite-binding substances was due almost entirely to pyruvic acid. Less quantitative estimations of pyruvic acid indicated a similar occurrence in thiamine-deficient rats. Lu (5) measured pyruvic acid and found increased amounts in the blood of thiamine-deficient rats, rabbits, and humans.

Sherman and Elvehjem (6) detected no rise in the bisulfite-binding substances of the blood of thiamine-deficient chicks, but they found nearly a 3-fold increase in the case of the cloacal ex-

\* From the thesis presented by Maurice E. Shils to the faculty of the School of Hygiene and Public Health, the Johns Hopkins University, in partial fulfillment of the requirements for the degree of Doctor of Science in Hygiene.



creta, the urinary component probably contributing the increased bisulfite-binding substances.

In view of these findings we were led to investigate the pyruvic acid and bisulfite-binding substances content of the urine of rats in various degrees of thiamine deficiency to determine whether an increase in these substances might afford a means of estimating (a) the degree of deficiency and (b) the effect of various factors and substances on the deficient animal. While engaged in this study, Banerji and Harris (7) published a paper describing the rapid and large increase of bisulfite-binding substances in the urine of thiamine-deficient rats and demonstrated that in these animals the decrease was proportional to the amount of thiamine administered. The work reported here confirms and extends their findings;<sup>1</sup> a preliminary note has appeared (8).

#### EXPERIMENTAL

*Urine Collection*—Single rats were placed in metabolism cages of the type used in this laboratory for mineral balance studies (10). Each cage rested on a funnel 11 inches in diameter at the rim. Directly below this large funnel was a sealed bulb supported in a small funnel by means of short projections. The bulb acted to separate the urine from the feces which were deflected into a metal cup, while the urine passed into a flask containing toluene.

The daily urinary output of normal young rats is quite small. Thiamine-deficient rats with the characteristic anorexia excrete even less. To avoid the loss of a goodly proportion of the urine through drying on the sides of the funnels, or the disadvantage of having to place several rats in the same metabolism cage and thus forfeiting individual data, a relatively large volume of urine was secured by incorporating sodium chloride in the diet at high levels. This allowed a daily collection of about 15 ml. of urine from a single rat eating as little as 3 gm. of diet. Loss of urine was relatively small and fairly constant, as shown by creatinine determinations. A 150 gm. rat fed a diet containing 9.4 per cent of sodium chloride may excrete as much as 60 ml. of urine daily.

<sup>1</sup> After the preparation of the manuscript of this paper was completed, Harper and Deuel (9) reported on the urinary excretion of pyruvic acid by thiamine-deficient rats. They found an increase as the deficiency progressed and an effect due to the quantity of food, as reported here.

*Effect of High Sodium Chloride Intake on Growth of Normal and Thiamine-Deficient Rats*—Cowgill *et al.* (11) found that the administration of large amounts of fluid by mouth to dogs on a vitamin B<sub>1</sub>-deficient diet markedly shortened the time required for the appearance of the anorexia characteristic of a lack of the vitamin. A washing out of the vitamin from the organism through elimination of the excess fluid is offered by these authors as a possible explanation of the results.

The following experiment was performed to determine whether feeding sodium chloride at a high level seriously affects the thiamine requirement, growth, and appearance. Four groups of rats, each containing six animals, were fed the following diets. Group 1 received high salt-low thiamine (Diet 1); Group 2 "normal" salt-low thiamine (Diet 2); Group 3 "normal" salt-adequate thiamine (Diet 3); and Group 4 high salt-adequate thiamine (Diet 4). Diet 2 was essentially Diet 112 of Arnold and Elvehjem (12). It consisted of sucrose 62, casein (acid-washed) 18, factor W  $\approx$  2 gm. of liver concentrate,<sup>2</sup> autoclaved peanuts<sup>3</sup> 10, autoclaved yeast<sup>4</sup> 4, Salts 51<sup>5</sup> 4, and percomorph oil 2 to 3 drops weekly per rat. The other diets were as follows: Diet 1, 10 gm. of sodium chloride to 100 gm. of Diet 2; Diet 2-B-300, 300  $\gamma$  of thiamine<sup>6</sup> to 100 gm. of Diet 2; and Diet 1-B-300, 300  $\gamma$  of thiamine to 110 gm. of Diet 1.

The figures for growth of the different groups are given in Table I.

The large amounts of sodium chloride received by Group 1 did not result in deficiency symptoms appearing any more quickly than in Group 2 receiving a "normal" small amount of the salt. There developed on Diet 1 a rather severe but chronic deficiency which was satisfactory for the needs of this study.

There is no significant difference between the weights of Groups 3 and 4. In both of these groups the animals were apparently

<sup>2</sup> The liver concentrate was obtained from The Wilson Laboratories through the courtesy of Dr. David Klein.

<sup>3</sup> The peanuts were autoclaved 10 hours.

<sup>4</sup> Northwestern yeast, autoclaved 2½ hours.

<sup>5</sup> CaCO<sub>3</sub> 1.5, KCl 1.0, NaCl 0.5, NaHCO<sub>3</sub> 0.7, MgO 0.2, Fe citrate 0.5, KH<sub>2</sub>PO<sub>4</sub> 1.7.

<sup>6</sup> The thiamine was furnished by Merck and Company, Inc., through the courtesy of Dr. R. Major.

healthy. Histological examination of the organs of the animals in Group 3 showed dilatation of the kidney tubules with no other changes. The same sort of dilatation is seen in kidneys of humans with diabetes insipidus where large quantities of water are being excreted. The kidneys of animals on the thiamine-deficient diet with high sodium chloride content showed no changes, evidently because the anorexia common to this group resulted in a very small urinary excretion. The results of this experiment permitted the conclusion that the use of sodium chloride at a 9.4 per cent level in the diet has no vitiating effects.

TABLE I

*Comparison of Growth of Rats Receiving Normal and High Amounts of Sodium Chloride with Optimal and Suboptimal Amounts of Thiamine*

Group No.	Diet No.	Average initial weight		Average weight after 5 wks. on diet		Average weight after 10 wks. on diet	
		♂	♀	♂	♀	♂	♀
		gm.	gm.	gm.	gm.	gm.	gm.
1	1. High salt-low thiamine	43		59*		86	
2	2. "Normal" salt-low thiamine	43		55 <sup>†</sup>		89	
3	2-B-300. "Normal" salt-optimum thiamine	49	44	139	111	198	151
4	1-B-300. High salt-optimum thiamine	45	44	118	99	182	144

\* One animal died with polyneuritis in the 6th week.

† One animal died with polyneuritis in the 8th week.

*Determination of Bisulfite-Binding Substances*—Satisfactory determinations may be made directly with aliquots of a 24 hour sample of rat urine diluted to 200 ml., although better end-points are obtained when adsorbing agents are previously employed. Experiments with adsorbents indicated that the use of Lloyd's reagent alone, the procedure applied to human urine (13), was not very satisfactory unless permutit was also used. The bisulfite-binding substances decreased after adsorption treatment, indicating the removal of some of these substances (not pyruvic acid). Increased sodium chloride concentration in rat urine has no effect

on the determination of bisulfite-binding substances provided the proper pH is attained.

The principle of the method of Clift and Cook is as follows: Pyruvic acid in acid solution reacts with bisulfite to form an addition compound which decomposes in a more alkaline medium. The liberated bisulfite is then measured with a dilute standard iodine solution, thus giving the concentration of pyruvic acid and other bisulfite-binding substances present.

We determined bisulfite-binding substances in rat urine as follows: 24 hour specimens are diluted to 200 ml. with distilled water. To each 25 ml. aliquot taken, 10 ml. of 0.125 N oxalic acid are added and then 0.6 ml. of approximately 1 M sodium bisulfite solution. After 15 minutes, 1 ml. of 1 per cent starch solution is added and the excess bisulfite is removed by adding 0.1 N iodine 2 to 3 drops in excess. In 4 to 5 minutes approximately 0.01 N sodium thiosulfate solution is used to remove the excess iodine; 0.005 N iodine solution is then added to the first faint color to remove the excess thiosulfate. 5.5 ml. of a clear saturated sodium bicarbonate solution are run in from a burette down the sides of the flask. 0.005 N iodine solution is immediately added dropwise from a microburette at a constant rate until a faint blue color spreads throughout the solution and persists for at least 4 to 5 seconds. The amount of standard 0.005 N iodine is a measure of the bisulfite-binding substances.

Numerous determinations showed that in the normal range of bisulfite-binding substances (in animals receiving adequate thiamine and with restricted food intake, a procedure adopted regularly after the first experiment), the difference in triplicate determinations seldom exceeded 0.05 ml. (1 drop) of 0.005 N iodine, a difference of 5 to 10 per cent. As the bisulfite-binding substances increased in thiamine-deficiency, the error increased slightly, but in 95 per cent of the determinations the differences remained within 0.2 ml. of 0.005 N iodine, still less than 10 per cent of the total bisulfite-binding substances.

In experiments in which it is desired to find the bisulfite-binding substances in rat urine after treatment with the adsorbents the following procedure is used: 125 ml. of the 200 ml. dilution of the 24 hour specimen are taken and 40 ml. of 0.125 N oxalic acid added. The solution is shaken for 4 to 5 minutes with a mixture of 10 gm.

of treated Lloyd's reagent (14) and 5 gm. of permutit, and then filtered. To 25 ml. aliquots of this filtrate 0.6 ml. of approximately 1 M sodium bisulfite solution is added and the procedure continued as described above, with the one difference that only 4 ml. of sodium bicarbonate solution are added. A small blank correction has to be made for bisulfite-binding substances in Lloyd's reagent.

A few workers (15, 16) stress the importance of cooling pyruvate solutions before titrating, although Clift and Cook (2) made no mention of this point. Several experiments in duplicate on aliquots of rat urine not adsorbed indicated that cooling at 10° gave no higher results than when the procedure was carried out at room temperature (25°). However, when the temperature reached 30°, lower values for bisulfite-binding substances were found. It is best, therefore, to keep the urine cool until ready for the final titration.

*Determination of Pyruvic Acid*—Pyruvic acid (Eastman) was redistilled *in vacuo*, and sodium pyruvate prepared as described by Peters (17) and kept in crystalline form until needed. Determinations as bisulfite-binding substance on the pyruvate gave 82 per cent of the theoretical value.

The estimation of pyruvic acid in urine was made by a slight modification of the method of Lu (1). To a 25 ml. aliquot of the 24 hour specimen of rat urine (diluted to 200 ml.) are added 10 ml. of 10 per cent trichloroacetic acid and the solution is filtered. 2 ml. of the filtrate are measured into a 15 ml. centrifuge tube and 2 ml. of the 2,4-dinitrophenylhydrazine solution are added. Lu's method is followed exactly except that 7 ml. of 1 N sodium hydroxide are added rather than 4 ml. The red color which develops is determined in a Klett-Summerson photoelectric colorimeter with Filter 54. The amount of pyruvic acid present is read from a standard curve constructed by plotting the colorimeter readings against various concentrations of pyruvate. We estimate the accuracy of this method to be within 5 per cent.<sup>7</sup>

*Diets*—In *Experiment 1* the diets all contained 9.4 per cent of sodium chloride. Diet 1, thiamine-low, has been given. Diets

<sup>7</sup> Lu (1) states that the method is fairly specific for pyruvic acid, although some other  $\alpha$ -keto acids may contribute to some extent, if present, to the colorimeter readings.

1-B-1, 1-B-2, and 1-B-3 contained 100, 200, and 300  $\gamma$  of thiamine respectively, per 110 gm. of Diet 1. In *Experiment 2* Diet 1-L was used. In this diet the sucrose of Diet 1 was replaced isocalorically by lard.

*Experiment 1. Bisulfite-Binding Substance and Pyruvic Acid in Progressive Thiamine Deficiency*

In this experiment the bisulfite-binding substances were estimated frequently during the time that the rats were restricted to the thiamine-low diet. Each experimental animal was paired with a control which received the same amounts of food supplemented with thiamine. The bisulfite-binding substances of each pair were measured at the same time. In most cases the determinations were made with and without treatment of the urine with the adsorbing agents. In addition, for some days before and after thiamine injection (toward the end of the experiment), pyruvic acid in the unadsorbed urine was measured as the 2,4-dinitrophenylhydrazone. Records of the food intake and weights of the animals were kept.

The results of representative animals are given in Figs. 1 and 2, depicting observations on deficient rats, and in Fig. 3, representing a control animal.

The experiment revealed the following.

In thiamine-deficient animals the bisulfite-binding substances and pyruvic acid in the urine increased greatly. The increase occurred before any symptoms of the deficiency were manifested.

As the deficiency progressed, the bisulfite-binding substances increased.

Within 24 hours after the injection of thiamine into the deficient animals the bisulfite-binding substances and pyruvic acid returned to normal.

There was a definite relation between food intake and bisulfite-binding substances and pyruvic acid. This was clearly indicated with animals receiving adequate thiamine and restricted to different amounts of food. Increasing or decreasing the food intake caused corresponding changes in bisulfite-binding substances and pyruvic acid. However, the changes in bisulfite-binding substances caused by varying the food intake were relatively small compared to the changes produced by the thiamine deficiency, except when

the food intake was very high (note the results on the 17th day, Fig. 1). When the deficient animals were given thiamine and access to an abundance of food, their food consumption increased greatly. Thiamine administration resulted in a decrease in the bisulfite-binding substances as normal carbohydrate metabolism was resumed; however, the rise due to increased food intake partly masked the decrease caused by the vitamin. This effect is clearly

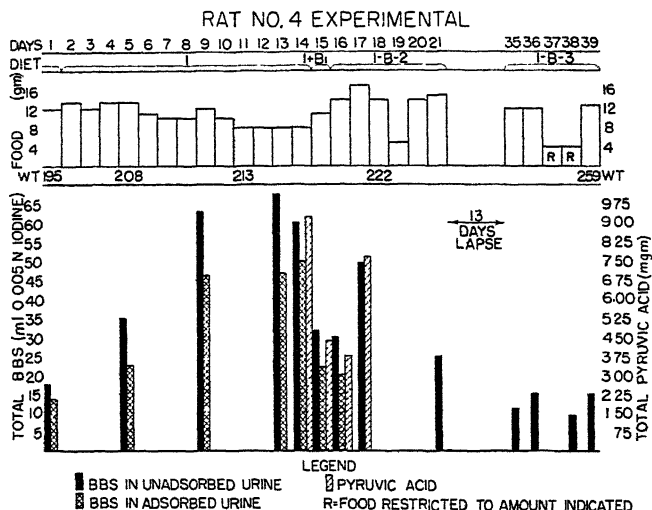


FIG. 1. Bisulfite-binding substances and pyruvic acid in the urine of a thiamine-deficient rat. Bisulfite-binding substances show a progressive rise as the deficiency progresses and a decrease on injection of thiamine (250  $\gamma$ , subcutaneously at the beginning of the 15th day). This animal was allowed to eat *ad libitum* after receiving the vitamin. Compare the levels of bisulfite-binding substance and pyruvic acid after thiamine administration with those of Rat 2 in Fig. 2, where the food was limited.

seen by comparing Figs. 1 and 2. The animal represented by Fig. 1 was allowed food *ad libitum* after receiving thiamine; that by Fig. 2 had its food intake restricted to the amount eaten before receiving the vitamin; consequently, the effect of the vitamin is much more clearly demonstrated.

These observations prompted the adoption of a procedure wherein the food intake of experimental and control animals was always restricted to the same small amount when determinations were to be made; this eliminated any effect of food intake.

When the food restriction procedure was adopted, it was found that the total bisulfite-binding substances of unadsorbed and adsorbed urine in all cases increased greatly in thiamine deficiency, in some cases reaching levels 10 times higher than the normal controls.

As Figs. 1 and 2 indicate, the bisulfite-binding substances and pyruvic acid both increased greatly in the deficient animals and

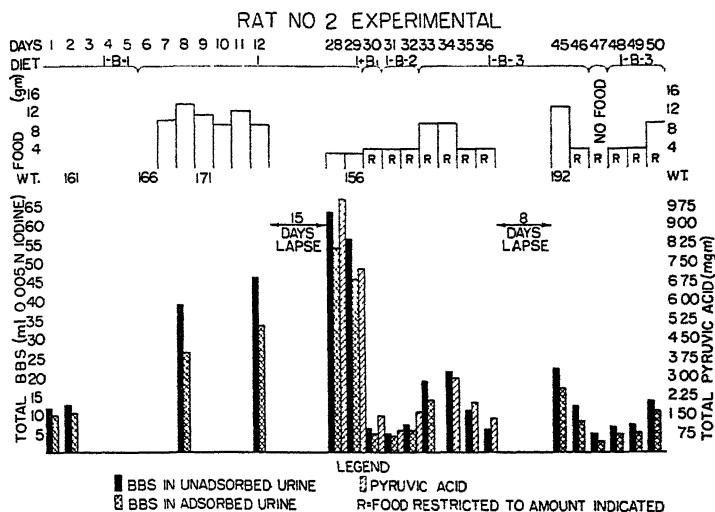


FIG. 2. Bisulfite-binding substances and pyruvic acid in the urine of a thiamine-deficient rat. 250  $\gamma$  of thiamine were injected subcutaneously at the beginning of the 30th day. Note the sharp drop in bisulfite-binding substances and pyruvic acid. This animal was paired with Rat 6 (Fig. 3) which received thiamine throughout and which was limited to the food intake of Rat 2. Note the fluctuation of bisulfite-binding substances and pyruvic acid as food intake is varied. There is an increased difference in the bisulfite-binding substances of urine which has been treated with adsorbing agents and that not treated.

immediately fell to normal values when thiamine was injected. The pyruvic acid concentration followed closely any changes in the bisulfite-binding level, strongly suggesting that it was the substance being measured as bisulfite-binding. This correlation extended to fluctuations produced by varying the food intake.

In an effort to answer the question of how much of the bisulfite-binding substance was due to pyruvic acid, the bisulfite-binding



substance (usually expressed in this paper as ml. of 0.005 N iodine) of the urine that had been adsorbed was calculated as mg. of pyruvic acid on the basis that 1 ml. of 0.005 N iodine = 0.22 mg. of pyruvic acid. These figures for the bisulfite-binding substance "pyruvic acid" were then compared with the values for pyruvic acid found by the hydrazone method. Table II shows this comparison. In animals receiving adequate thiamine practically all of the bisulfite-binding substance can be accounted for as pyruvate. At the higher concentrations found in deficient animals, the values

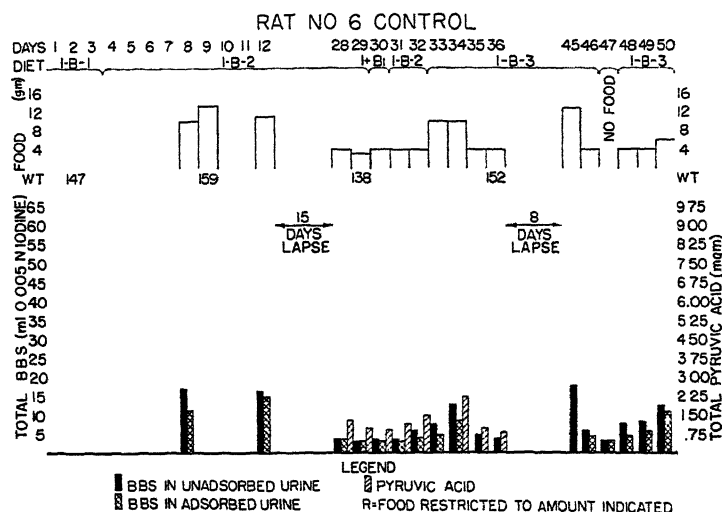


FIG. 3. Bisulfite-binding substances and pyruvic acid in the urine of a rat receiving adequate thiamine.

usually exceeded those of the hydrazone pyruvic acid, but by no more than 10 to 20 per cent. The changes occurring with varying food intake are paralleled by changes in pyruvic acid. It appears, then, that a very great part, if not all, of what is measured as bisulfite-binding substances in urine which has been treated with adsorbing agents is pyruvic acid.

Fig. 2 indicates the differences between the bisulfite-binding levels determined on urine treated with and without adsorbing agents. At low concentrations the differences are quite small; as the bisulfite-binding substances increase, so do the differences.

As the non-adsorbable pyruvic acid increases, so does some other adsorbable substance or substances. Investigation was made of some substances which might conceivably account for this increase.

TABLE II  
*Comparison between Levels of Bisulfite-Binding Substances (B.B.S.)  
(Calculated As Pyruvic Acid) and Pyruvic Acid Determined As  
2,4-Dinitrophenylhydrazone*

Rat 1				Rat 2				Rat 3				Rat 4			
Day	B.B.S. "pyruvic acid"	Hydrazone pyruvic acid	Pyruvic B.B.S.	Day	B.B.S. "pyruvic acid"	Hydrazone pyruvic acid	Pyruvic B.B.S.	Day	B.B.S. "pyruvic acid"	Hydrazone pyruvic acid	Pyruvic B.B.S.	Day	B.B.S. "pyruvic acid"	Hydrazone pyruvic acid	Pyruvic B.B.S.
Deficient animals															
21	7.8	7.0	89.9	28	11.9	10.0	89.1	34	8.8	7.0	79.6	13	11.0	9.3	84.5
22	9.1	7.9	86.9	29	10.0	7.3	73.0	35	6.3	4.9	77.8	14*	4.9	4.4	89.9
23	7.6	7.0	92.1	30*	1.1	1.4	127.3	38*	1.4	1.1	78.7	15*	4.4	3.8	86.6
24	7.6	7.6	100.0	31*	1.0	1.1	110.0	39*	1.6	1.4	87.7				
25	7.0	6.2	88.7	32*	1.2	1.6	133.3	40*	2.1	2.0	95.4				
27*	4.8	4.6	95.9					41*	3.6	3.1	86.2				
								42*	2.1	2.0	95.4				
Control animals															
Rat 5				Rat 6				Rat 7				Rat 8			
22	1.8	2.2	122.2	28	1.1	1.3	118.2	34	1.7	1.4	82.5	13	2.4	2.1	87.6
23	1.4	1.5	107.1	29	0.7	1.0	143.0	35	1.7	1.3	76.6	14	2.2	2.0	91.0
24	1.2	1.5	124.9	30	0.7	0.8	114.2	36	1.4	1.0	71.5	15	2.6	3.5	134.7
25	3.6	3.5	97.4	31	0.8	1.1	137.8	38	0.9	0.8	89.9				
26	2.9	2.7	93.2	32	1.0	1.4	140.0	39	0.8	0.7	87.6				
27	3.1	3.2	103.2					40	1.7	1.3	76.6				
								41	2.6	2.2	84.8				
								42	0.8	1.1	137.8				

\*Thiamine administered.

Uric acid binds small amounts of bisulfite, reduces iodine, and is adsorbed on Lloyd's reagent. Quick (18) found that in humans pyruvic acid had a very marked stimulatory effect on the excretion of uric acid. Since in thiamine deficiency pyruvic acid concentration in the tissues increases, an increased excretion of uric acid

or of allantoin might be expected. Investigation disclosed the following. (a) Allantoin does not bind bisulfite nor reduce iodine and cannot be responsible for the increased bisulfite-binding substance of unadsorbed urine.<sup>8</sup> (b) There is no significant difference in the uric acid excretion of thiamine-deficient and control rats. The uric acid was determined by the method of Benedict and Hitchcock (19) and was always less than 0.4 mg. in 24 hours. (c) Creatine, which does not bind bisulfite, was increased in thiamine-deficient animals. However, the increase is not directly attributed to the deficiency, but rather can be accounted for by the degree of starvation induced by the characteristic anorexia.

*Experiment 2. Effect of Fat on Bisulfite-Binding Substances of Normal and Thiamine-Deficient Rats*

The "sparing action" of fat on thiamine is well known (20-22). Stirn, Arnold, and Elvehjem (22) found that the isocaloric substitution of fat for sucrose allowed polyneuritic rats to grow as well as those given thiamine. It was of interest, therefore, to find the effect of fat on the bisulfite-binding level.

Rats were made thiamine-deficient by being fed Diet 1. Lard was then substituted isocalorically for sucrose in a diet (No. 1-L) fed to the deficient rats as well as to those receiving thiamine. The lard content of Diet 1-L was 36.6 per cent. The carbohydrate content was very low.

The animals were given the same limited amount of food on the day of urine collection and on the preceding day unless otherwise stated. 2 and 2.67 gm. of Diet 1-L have the same caloric value as 3.00 and 4.00 gm., respectively, of Diet 1. The urine was not treated with adsorbents.

The bisulfite-binding substances of five control animals receiving adequate thiamine (first on the high sugar, then on high fat diets) remained within the normal range (below 8 ml. of 0.005 N iodine per 24 hour urine sample). All of the five deficient animals showed very high bisulfite-binding values (25 to 44 ml. of 0.005 N iodine) on the high sucrose, thiamine-deficient diet. All showed some decrease with the feeding of the high fat diet, but the level remained definitely elevated in four of the five until thiamine was

<sup>8</sup> Harper and Deuel (9) state, in an objection to the use of the procedure for bisulfite-binding substances, that allantoin binds bisulfite. We do not find this so by our procedure.

given 2 to 3 weeks later. The fifth rat of the deficient group (Rat 26) showed a return to normal values within 2 weeks without thiamine. Sex did not seem to influence the results.

The change from a high carbohydrate to a high fat diet stopped the weight loss in the deficient animals and allowed them to gain in weight and improve in appearance.

The bisulfite-binding values for a normal control animal and for two of the deficient rats are given in Table III. Rat 41 is repre-

TABLE III

*Effect of Fat on Bisulfite-Binding Substances (B.B.S.) of Normal and Thiamine-Deficient Rats*

Bisulfite-binding substances expressed as ml. of 0.005 N iodine per 24 hour urine sample.

Rat 45, ♂				Rat 41, ♂				Rat 26, ♂			
Day	Diet No.	Amount fed*	B.B.S.	Day	Diet No.	Amount fed	B.B.S.	Day	Diet No.	Amount fed	B.B.S.
		gm.				gm.				gm.	
1	1-B-3	4.0	4.7	1	1	4.0	44.0	1	1	4.0	37.7
2	1-L + B <sub>1</sub>	2.67	3.7	2	1-L	2.67	29.4	2	1	4.0	37.0
3	1-L + B <sub>1</sub>	2.67	4.5	3	1-L	2.67	30.6	3	1-L	2.0	25.3
8	1-L + B <sub>1</sub>	2.67	5.2	8	1-L	2.67	32.1	8	1-L	2.0	9.7
14	1-L + B <sub>1</sub>	2.67	7.9	14	1-L	2.67	30.8	9	1-L	2.0	9.4
15	1-L + B <sub>1</sub>	2.67	7.8	15	1-L	2.67	25.1	16	1-L	2.0	5.8
16	1-L + 250 γ B <sub>1</sub> †	2.67	4.7	16	1-L + 250 γ B <sub>1</sub> †	2.67	5.0	17	1-L + 200 γ B <sub>1</sub> †	2.0	2.5
17	1-L + 250 γ B <sub>1</sub> †	2.67	4.4	17	1-L + 100 γ B <sub>1</sub> †	2.67	4.2				

\* The amount of food indicated is the amount allowed on the day in the metabolism cage and the day preceding. On other days the animals were allowed to eat *ad libitum*.

† Thiamine was injected subcutaneously at the beginning of the 24 hours in the metabolism cage.

sentative of the four deficient animals which showed continued high bisulfite-binding substances on the high fat diet. Rat 26 is the animal showing a return to high normal without thiamine.

#### DISCUSSION

The results presented here, together with those of Banerji and Harris (7), seem to leave no doubt that the level of bisulfite-binding substances and pyruvic acid in the urine of rats is a sensi-

tive indicator of the state of thiamine nutrition. The increased concentration of the specific metabolite accumulating as a result of the specific biochemical lesion may well be taken as a criterion of the deficiency.

The effect of the quantity of food ingested on the amount of bisulfite-binding substances and pyruvic acid excreted possibly can be related to the degree of carbohydrate storage and utilization, although direct evidence for this view is lacking. We have found that with a deficient animal showing a high excretion of bisulfite-binding substances there will be a reduction when food is withheld or continued at the same low level for several days. Increasing the food allowance to a partially fasted normal rat results in an increase in bisulfite-binding substance and pyruvic acid.

The last fact would make it appear that the kidney threshold for pyruvic acid is low. This is borne out by the presence of pyruvic acid in the urine of rats receiving adequate thiamine and only limited amounts of food. Because of the low threshold, any accumulation of pyruvic acid through thiamine deficiency in the body will immediately manifest itself by increased urinary excretion.

On the basis of this view it would be expected that the feeding of any substance that increases the amount of pyruvic acid formed would result in its increased excretion by deficient or normal animals. This is supported by the results of Banerji and Harris (7) who fed lactate to normal and thiamine-deficient rats. There were not only large increases in excretion of bisulfite-binding substances from the deficient animals but also a small increase in the animals receiving optimum amounts of the vitamin. Only a small increase would be expected in normal animals, since they metabolize pyruvic acid very quickly (23).

The relationship between bisulfite-binding substances and pyruvic acid in both blood and urine seems rather clear in thiamine deficiency in rats and pigeons. Most, if not all, of the bisulfite-binding substances can be accounted for as pyruvic acid. However, in human deficiency the relationship is not so clear. Wilkins *et al.* (24) found a much greater increase in blood bisulfite-binding substance than can be accounted for by the rise in pyruvic acid. Furthermore, Platt and Lu (25) frequently found that blood

pyruvic acid could be restored to normal levels by thiamine administration, while at the same time there was only a slight reduction in the amount of bisulfite-binding substances. Possibly the experimental procedures were at fault, or other deficiencies entered into the picture, or else the human reacts differently from the other species studied.

The effect of fat in improving the physical condition of the deficient rats was as expected from previous work. It would seem reasonable to expect a concomitant quick return to normal of the urinary bisulfite-binding substance when the animals no longer had to metabolize dietary carbohydrate. However, after 2 to 3 weeks on the high fat diet the bisulfite-binding substances were still highly elevated in four of the five deficient animals, although the levels were less than on the high carbohydrate diet. There are certain possible explanations. (a) The small intake of carbohydrate (about 0.12 gm. per rat per day) on Diet 1-L together with that derived from protein may be enough to cause accumulation of bisulfite-binding substance as long as the animal is deficient, even though it gains in weight. (b) When large amounts of fat are being metabolized in the absence of thiamine, intermediary bisulfite-binding products are formed.

The fact that one animal had a return to normal of the bisulfite-binding substances may be an indication that given sufficient time all animals would have behaved similarly.

Banerji (26) has shown that feeding a high fat-thiamine-low diet to rats does not result in increased excretion of bisulfite-binding substances as does a high sucrose-thiamine-low diet. In his experiments, since the animals were transferred from a ration with adequate thiamine to the high fat-low thiamine diet, they had a store of the vitamin in their tissues to begin with. In this important respect, therefore, the experiment differs from that reported here. It would be of great interest to know the thiamine content of rats at the end of an experiment such as that described by Banerji. It may be that the animal retains enough of the vitamin to allow normal excretion of bisulfite-binding substances since very little seems to be required on a high fat diet.

We have confirmed the fact that under the conditions described for determining bisulfite-binding substance acetone is not measured. Acetoacetic acid, however, is reported (2) to bind bisulfite

under similar conditions, although not quantitatively. Despite the long period on the high fat diet, the control animals receiving thiamine showed no elevated bisulfite-binding substance, thus eliminating the possibility that the increase in the deficient animals is caused by a ketosis due to the high fat-low carbohydrate diet alone. The quick response to thiamine indicates that the vitamin deficiency was responsible for the elevated values.

The results reported here together with those of Banerji and Harris (7) invite further investigation on the possibilities for assay procedure based on urinary levels of bisulfite-binding substance or pyruvic acid. In a future paper we will report on the behavior of bisulfite-binding substances in the urine of rats receiving various levels of the vitamin.

#### SUMMARY

1. Procedures are given for the determination of bisulfite-binding substances in the urine of rats.

2. A rapid increase in bisulfite-binding substances occurs in the urine of rats on a thiamine-low diet, the rise being proportional to the degree of deficiency and occurring before any other symptoms associated with the deficiency. Administration of thiamine brings the level of bisulfite-binding substances to normal within 24 hours.

3. The level of pyruvic acid accounts quite closely for the substance (or substances) measured as bisulfite-binding.

4. The amount of food intake has a marked influence on bisulfite-binding substances and pyruvic acid concentration in urine.

5. The isocaloric substitution of fat for sucrose in the diet fed to thiamine-deficient rats results in improved growth, but only in a partial return to normal in urinary bisulfite-binding substances. In four of five deficient animals the bisulfite-binding substances still remained abnormally high until thiamine was given.

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## TRANSFER OF RADIOACTIVE SODIUM ACROSS THE PLACENTA OF THE CAT\*

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The placentae of various mammals have been classified by Grosser (1) into four groups. Differentiation among these groups is based upon the nature and number of tissue layers placed between the maternal and fetal circulations. It is the purpose of the present series of investigations to study the passage of substances across these different kinds of placentae throughout the gestation period and to correlate the findings with the morphology of the placenta. Observations with radioactive sodium have demonstrated that the use of isotopes provides the direct and relatively simple approach demanded by the problem.

The results on the placenta of the guinea pig, in which maternal blood is separated from fetal by only two tissue layers (chorionic epithelium and endothelium of fetal blood vessels; hemochorial type of placenta) have already been presented (2). The present study has to do with measurement of the rate of transfer of radioactive sodium across the placenta of the cat, in which the maternal and fetal circulations are separated by four layers (endothelium of maternal blood vessels, chorionic epithelium, chorionic mesenchyme, and endothelium of fetal blood vessels; endotheliochorial type of placenta).

### EXPERIMENTAL

#### *Procedure*

Twelve pregnant cats, which yielded forty fetuses, were used for the experiments. The pregnant animals were lightly etherized

\* Aided by a grant from the Rockefeller Foundation Fluid Research Fund of the School of Medicine, the Johns Hopkins University.

and about 3 cc. of an isotonic solution, containing  $\text{Na}^{24}$ , were injected into a foreleg vein. Anesthesia was immediately discontinued after the injection. After a time interval fixed by the purpose of the experiment, the animal was again etherized, the fetuses delivered by cesarean section, and a sample of heart blood taken from the mother. The placentae were separated and weighed after removal of excess blood. The fetuses were weighed and then ashed at red heat with sulfuric acid. Measurements of the radioactivities of the fetal ash and maternal plasma were made with the pressure ionization chamber-string electrometer circuit previously described (2). The measured radioactivity was corrected for background, absorption by the sample, and radioactive decay in the manner already given (2).

The samples of  $\text{Na}^{24}$  were prepared with the high voltage generator of the Department of Terrestrial Magnetism, Carnegie Institution of Washington (3). The samples were not used for at least 24 hours after preparation to allow time for the disappearance of any  $\text{Cl}^{38}$ .

The amount of  $\text{Na}^{24}$  chosen for injection was such that samples taken from the animal would exceed a lower limit set by the precision of the measuring apparatus and would be within an upper limit fixed by the tolerance of the animal to radioactive material; i.e., less than about 1 microcurie ( $3.7 \times 10^4$   $\beta$ -rays per second) per cc. of animal (4-6). With the pressure ionization chamber-string electrometer, the precision of electrical measurements and convenience from point of view of the time necessary for a measurement required a sample strength of not less than 100  $\beta$ -rays per second (2) for an over-all precision of 4 per cent in the method. The amounts of  $\text{Na}^{24}$  injected were chosen to meet this requirement; they were always far below the upper limit of 1 microcurie per cc.

To obtain an adequate sample strength, the amount of  $\text{Na}^{24}$  taken for injection can be calculated from the relationship, total  $\text{Na}^{24}$  injected = the desired amount in the sample  $\div$  the dilution factor. In the present experiments, since measurements of plasma samples and fetuses were made, it was necessary to refer to both of these to determine the minimal quantity of  $\text{Na}^{24}$  for an experiment.

$\text{Na}^{24}$ , injected intravenously, quickly comes to equilibrium

with the extracellular fluids. It is finally distributed equally throughout a volume approximately 25 per cent of the body volume (2, 7, 8). Its equilibrium concentration in the plasma is therefore 4 times that in the body. Plasma samples of 2 gm. were taken for measurement. The dilution factor in this instance is 4 times the weight of the plasma sample (about 2 gm.)  $\div$  the total weight of the animal. To obtain a plasma concentration of  $\text{Na}^{24}$  yielding 100  $\beta$ -rays per second in a 2 gm. sample and in a cat weighing 5000 gm., the amount of  $\text{Na}^{24}$  injected would be  $(5000 \text{ gm.} \div 2 \text{ gm.} \times 4) \times 100 \text{ } \beta\text{-rays per second} = 63,000 \text{ } \beta\text{-rays per second}$ .

In the fetus at equilibrium with the mother in regard to  $\text{Na}^{24}$ , the dilution factor is approximately the ratio of the fetal to the maternal weight (the concentration factor introduced by the difference in extracellular fluid volumes being neglected). In rate measurements on the fetus, the fetal  $\text{Na}^{24}$  concentration is usually about one-twelfth of the equilibrium concentration and the dilution factor is one-twelfth of the ratio of the weight of the fetus to the weight of the mother. The amount of  $\text{Na}^{24}$  taken for injection (about 3 microcuries) exceeded the quantity calculated from the dilution factor by 25 to 50 per cent. This compensated for the reduction of radioactivity due to absorption of  $\beta$ -rays by the plasma or fetal ash (2). With fetuses of low weight, several members of a litter were pooled into a single sample in order to conserve  $\text{Na}^{24}$ .

Before an experiment, the size of the fetuses was estimated by palpation and, when necessary, by lateral x-ray photographs; calculation of the probable weight was made from the crown-rump length, with Coronios' data (9).

*Units*—The activity of the radioactive material is expressed in terms of  $\beta$ -particles per second. A sample of 100  $\beta$ -rays per second is one that gives a deflection rate equivalent to a  $\beta$ -ray point source emitting 100  $\beta$ -rays per second in all directions and situated at the standard measuring position in our apparatus. 1 microcurie is about 37,000  $\beta$ -rays per second in these units.

All quantities and concentrations of  $\text{Na}^{24}$  found in the fetus have been corrected to unit concentration in the corresponding maternal plasma. The term "corrected" placed after a value for  $\text{Na}^{24}$  found in the fetus means, therefore, that the measured

value in the fetus has been divided by the measured value per cc. of maternal plasma; *i.e.*, values in the fetus have been corrected to a concentration of one  $\beta$ -ray per second per cc. of maternal plasma.

### Results

*Establishment of Equilibrium between Maternal Plasma and Fetus*—In order to plan experiments to determine the rate of transfer of  $\text{Na}^{24}$  across the placenta, it is necessary to know the shape of the curve describing the establishment of equilibrium between the maternal plasma and fetus. In these experiments

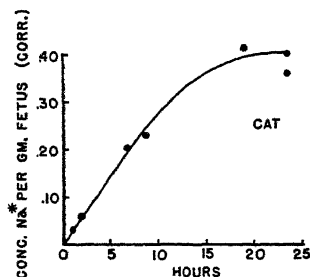


FIG. 1

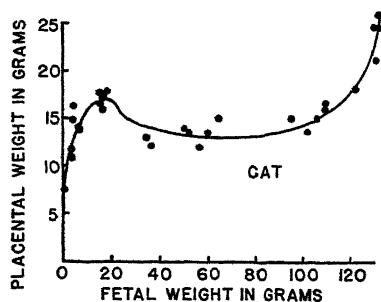


FIG. 2

FIG. 1. Rate of equilibration of  $\text{Na}^{24}$  in fetus with  $\text{Na}^{24}$  in maternal plasma. All fetuses weighed 95 gm. or more. The concentration of  $\text{Na}^{24}$  per gm. of fetus has been corrected to a concentration of one  $\beta$ -ray per cc. of maternal plasma as explained above in the section on "Units."

FIG. 2. Variation of placental weight with change of fetal weight.

with the cat, unlike those with the guinea pig (2), no apparent error was introduced by delivering the several members of a litter at different time intervals after the injection of  $\text{Na}^{24}$  into the maternal circulation.

The data of Fig. 1 indicate that fetuses of 95 gm. or greater come to within 10 per cent of a limiting equilibrium value with respect to  $\text{Na}^{24}$  in the maternal plasma only after 12 to 18 hours. This is to be contrasted with the extracellular fluid which comes to within 10 per cent of equilibrium with the plasma in about 4 minutes (2, 10). The concentration of  $\text{Na}^{24}$  in the fetus appears to increase linearly for at least 5 hours after intravenous injection

into the mother. Measurement of the increase in concentration of  $\text{Na}^{24}$  up to this time consequently forms a reliable criterion of the rate of transfer of  $\text{Na}^{24}$  across the placenta. In view of these findings, the routine procedure for determination of placental transfer rates has been to remove fetuses 1 hour after the injection of  $\text{Na}^{24}$ .

It is to be noted in Table I that the equilibrium concentration of  $\text{Na}^{24}$  is greater in small than in large fetuses (2).

*Rates of Placental Transfer*—The experiments have been planned to measure the rates of placental transfer at different parts of the

TABLE I  
*Data Necessary for Calculation of Safety Factor, Rate of Na Transfer to Fetus, and Rate of Na Accretion by Growing Fetus*

Fetal weight	$\text{Na}^{24}$ transferred per gm. fetus per hr. (corrected)*	Daily weight increase	Equilibrium concentration of $\text{Na}^{24}$ per gm. fetus (corrected)*	Safety factor	Total Na transferred per hr. to fetus	Total Na retained in hourly growth of fetus
(1)	(2)	(3)	(4)	(5)	(6)	(7)
<i>gm.</i>		<i>per cent</i>			<i>mg.</i>	<i>mg.</i>
130	0.031	5.1	0.37	39	13.5	0.34
40	0.052	10.5	0.50	24	6.9	0.29
10	0.081	17.5	(0.55)	20	2.7	0.13
1	0.11	25	(0.6)	18	0.36	0.021
0.15	0.17		(0.6)		0.08	

The Na concentration in the maternal plasma has been assumed to be 3.3 mg. per cc. for the calculation of Columns 6 and 7. Extrapolated values are in parentheses. Underlined figures are uncertain.

\* The term "corrected" is explained in the text in the section on "Units."

gestation period. In all experiments, the placentae, following removal of excess blood, and fetuses have been weighed, and measurement has been made of the concentration of  $\text{Na}^{24}$  in the maternal plasma and of the total quantity of  $\text{Na}^{24}$  in the fetus (each sample was taken at a known interval, about 1 hour after intravenous injection into the mother). Such measurements can be expressed in terms of total transfer to a fetus per unit of time, of transfer rate per unit weight of fetus, or of transfer rate per unit weight of placenta. The data necessary for deriving these relations are presented graphically. Fig. 2 gives the data

on changes in placental weight with fetal weight (fetal weight can be translated into gestation age by reference to Fig. 4). The peak in the placental weight-fetal weight curve in the earlier stages of pregnancy appears to have its counterpart in the sheep (11). The change in total hourly transfer of  $\text{Na}^{24}$  to the fetus with change of fetal size is presented in Fig. 3.

The data of Figs. 2 and 3 have been used to construct the curves of Figs. 4 and 5. In Fig. 4 are shown the changes in rate of transfer across a unit weight of placenta throughout as much of intrauterine life as it has been feasible to investigate. As is clear

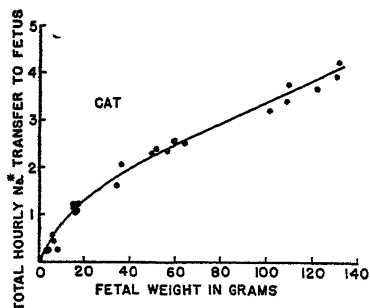


FIG. 3

FIG. 3. Variation of total hourly transfer of  $\text{Na}^{24}$  to fetus with change of fetal weight.

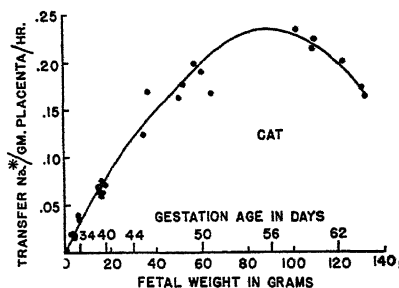


FIG. 4

FIG. 4. The gestation age has been estimated from the fetal weight by reference to the data of Coronios (9). The weight of the smallest fetus on the graph was 0.15 gm., corresponding to a gestation age of 15 to 20 days; the transfer rate per gm. of its placenta was 0.004 unit.

from the curve, the transfer rate per unit weight of placenta makes a striking over-all increase with age. Thus at 15 to 20 days the transfer rate is about 0.004 unit per gm. of placenta, while at 57 days it is 0.24 unit. Transfer of  $\text{Na}^{24}$  across a unit weight of the 57 day placenta is consequently about 60 times as rapid as across a unit weight of the 15 to 20 day placenta. As is clear from the curve, the findings indicate a decrease in transfer rate per unit weight of placenta from about the 55th day to term.

Fig. 5 gives the rate at which  $\text{Na}^{24}$  is supplied from the maternal plasma to each gm. of fetus as this rate varies with fetal weight. This rate is high in the early fetus and falls with increase of gesta-

tion age. For example, the ratios of rates of transfer for gestation ages of 15 to 20 days, 40 days, and 60 days are 5.5:2.3:1. The high rate in the early fetus, in spite of a low transfer rate per gm. of placenta, is accounted for by the relatively large ratio of placental to fetal weight. Fig. 5 also shows the daily per cent weight increase of the fetus during that part of pregnancy under consideration. This has been calculated (2) from the data of Coronios (9). It is apparent from Fig. 5 that the transfer rate and growth rate curves run parallel.

*Rate of Na Supply Compared to Fetal Need*—It is possible to calculate, with knowledge of the transfer rate of  $\text{Na}^{24}$ , of the

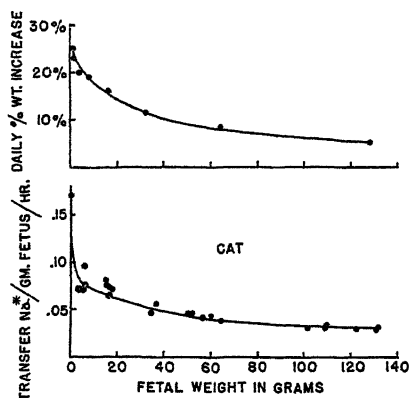


FIG. 5. Comparison of curve of daily per cent weight increase and curve of transfer rate of  $\text{Na}^{24}$  per gm. of fetus during the gestation period.

concentration of  $\text{Na}^{24}$  in the fetus when at equilibrium with unit  $\text{Na}^{24}$  concentration in the maternal plasma, and of the growth rate, by what factor the Na supply to the fetus exceeds the demands of growth. This factor has been termed the safety factor (2). It has been shown that the safety factor = the transfer of  $\text{Na}^{24}$  per gm. of fetus per hour (corrected)  $\times 24 \times 100 \div$  the equilibrium concentration of  $\text{Na}^{24}$  per gm. of fetus (corrected)  $\times$  daily per cent increase in weight.

If a reasonable value for the concentration of Na in the maternal plasma is taken, the rates of Na transfer to the fetus and incorporation of Na by the fetus may be directly calculated. Express-



sions for these rates have previously been derived (2). The rate at which Na is supplied to the fetus is total  $\text{Na}^{24}$  transfer per hour (corrected)  $\times \text{Na}_{m.p.}$ , where  $\text{Na}_{m.p.}$  represents the concentration of Na in the maternal plasma. The hourly accretion of Na by the growing fetus is equilibrium concentration of  $\text{Na}^{24}$  per gm. of fetus (corrected)  $\times \text{Na}_{m.p.} \times \text{fetal weight} \times \text{daily per cent increase in weight} \div 24 \times 100$ .

The sodium safety factor, amount of Na transferred per hour, and the amount retained in hourly growth are given in Table I for fetuses of several sizes. It is apparent that the safety factor increases with increase of fetal age.

#### DISCUSSION

The placenta of the cat is adapted by changes in its weight and in transfer rate per unit weight to the requirements of the fetus during the gestation period. The more important of these two changes is in the unit transfer rate. From approximately the 17th day until term, the weight of the placenta increases 5 times, whereas the maximum change in unit transfer rate of  $\text{Na}^{24}$  over the same period is of the order of 60 times.

Placental weight and unit transfer rate change in such a way that the curve describing the transfer of  $\text{Na}^{24}$  per unit weight of fetus, during that part of pregnancy studied, parallels the growth curve of the fetus. This is a phenomenon already noted in the guinea pig (2) and it strengthens the tentative hypothesis that the fundamental principle underlying placental transfer to a unit weight of fetus is that it shall parallel the growth rate of the fetus.

The several factors which may alter the transfer rate across the placenta have already been discussed (2). It has been pointed out that from the point of view of circulatory changes, the only suggestive evidence to account for the increase in unit transfer rate which occurs with increase of gestation age has come from studies on the sheep (11). In the sheep it has been found that the rate of blood flow through the fetal vessels of the placenta about doubles during the last half of pregnancy.

Histological changes, observed in the placenta with progress of gestation, appear, however, to be of clearer meaning. The placenta of the cat, in the classification of Grosser (1), is of the endotheliochorial type; *i.e.*, endothelium of maternal blood vessels, chorionic epithelium, and connective tissue, and endothelium of

fetal blood vessels are interposed between the two circulations. Aging of the placenta is accompanied by changes in the chorionic epithelium. This epithelium consists, in early forms, of two distinct layers, the outer of which is syncytial (nuclei are present but cell boundaries cannot be seen). As pregnancy advances, the inner epithelial cells progressively diminish in height and then become discontinuous, so that in many places the chorionic epithelium consists of but a single layer, the syncytium. The increase in transfer rate per unit weight of placenta can consequently be correlated with the thinning of the chorionic epi-

TABLE II  
*Comparison of Placentae of Cat and Guinea Pig*

Period of gestation, tenths of total	Average fetal age for indicated period		Average fetal weight		Average placental weight		Na <sup>24</sup> transferred per gm. placenta per hr.		Ratio of transfer rate per gm. placenta, guinea pig to cat	Ratio of placental weights, cat to guinea pig
	Cat	Guinea pig	Cat	Guinea pig	Cat	Guinea pig	Cat	Guinea pig		
	days	days	gm.	gm.	gm.	gm.	units	units		
0.3-0.4	21.7	23.8	0.3	1	7	1	0.004	0.18	43	7
0.4-0.5	27.9	30.6	2	2	11	1.4	0.01	0.3	30	8
0.5-0.6	34.1	37.4	6	7	14	2.2	0.024	0.85	36	6
0.6-0.7	40.3	44.2	18	17	17	2.7	0.072	1.4	20	6
0.7-0.8	46.5	51	42	34	13.5	3.1	0.15	0.70	4.7	4
0.8-0.9	52.7	57.8	73	64	13	3.7	0.23	1.85	8	3.5
0.9-1.0	58.9	64.6	110	94	15.5	4.7	0.21	1.85	9	3.3

thelium which accompanies advance of pregnancy. This is suggestive evidence that changes in placental transfer rates are in considerable part due to changes in placental permeability.

The placenta of the guinea pig has previously been studied (2) with radioactive sodium in the same way as that of the cat. The gestation periods of the cat and guinea pig are of about the same duration (about 62 days for the cat and 68 days for the guinea pig). Moreover the growth rates of cat and guinea pig fetuses are not dissimilar and in Table II it can be seen that the two kinds of fetuses when of comparable age are of about the same weight. Hence it is possible to compare the two types of placentae at almost equivalent parts of pregnancy.

The guinea pig placenta, unlike that of the cat with its four

tissue layers, has only chorionic epithelium and endothelium of fetal blood vessels placed between the fetal and maternal blood, and in late stages the chorionic epithelium disappears. All other factors being more or less the same, it might be anticipated that the rates of transfer across unit weights of these two types of placentae would be correlated with their structural differences. This appears to be true. At all stages of gestation a unit weight of guinea pig placenta has a transfer rate many times that of the cat (Table II). Thus in the third tenth of pregnancy, transfer of  $\text{Na}^{24}$  across a unit weight of the guinea pig placenta is 43 times as rapid as across a unit weight of the cat placenta; this factor diminishes with the duration of gestation and reaches a value of about 9 in the last tenth of the gestation period. It is therefore clear that in these two types of placentae, large differences in unit transfer rate can be correlated with known histological differences.

It is evident from Table II that the "late" cat placenta has a unit transfer rate of the same magnitude as the "early" guinea pig placenta. This finding also appears to have a histological basis. A single layer of chorionic epithelium is present in the guinea pig placenta of fairly early stages; this later thins and probably disappears. The early cat placenta has two layers of chorionic epithelium, whereas in late stages there is only a single layer. From the point of view of chorionic epithelium, the early guinea pig and late cat placentae are therefore similar and this similarity appears to be reflected in the unit transfer rates.

The relatively low transfer rate per unit weight of placenta in the cat is compensated for by a relatively heavy placenta. Thus the weight of the cat placenta in early stages is 7 times, and in later stages over 3 times that of the guinea pig placenta (Table II). The compensation through increased mass of the placenta is, however, not complete. This is apparent from the total transfer rate of Na to the fetus which on the average is twice as high in the guinea pig as in the cat (Table I). In turn, the safety factor (ratio of Na retained by the fetus in growth to Na supplied the fetus) has an average value of about 25 in the cat (Table I) whereas the average in the guinea pig is 50.

We are much indebted to Dean B. Cowie of the National Cancer Institute for making the sodium bombardments with the Carnegie generator.

## SUMMARY

1. The cat fetus comes to within 10 per cent of equilibrium with  $\text{Na}^{24}$  in the maternal plasma only after 12 to 18 hours. This is in striking contrast to the maternal extracellular fluid which comes to within 10 per cent of equilibrium with intravenously injected  $\text{Na}^{24}$  in about 4 minutes.

2. Changes in the rate of placental transfer per unit weight of placenta have been measured with  $\text{Na}^{24}$  from a gestation age of 15 to 20 days until term. The unit transfer rate of  $\text{Na}^{24}$  increases 60 times during this period.

3. The shape of the relative growth curve of the cat fetus is similar to that of the curve describing the change of rate of transfer of  $\text{Na}^{24}$  to a unit weight of fetus at different periods of pregnancy.

4. The fetus receives across the placenta an average of about 25 times as much Na as is incorporated in the growing tissues.

5. Differences in unit transfer rate across the cat and guinea pig placentae at comparable stages of pregnancy are analyzed on the basis of the known histological differences of the two types of placentae. It is shown that differences in transfer rate can be correlated with the histological differences.

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## MOVING BOUNDARY ELECTROPHORETIC STUDY OF INSULIN\*

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The present investigation was undertaken to explore the behavior of insulin by the Tiselius moving boundary electrophoretic technique. We wished in particular to ascertain whether or not crystalline insulin exhibits the single sharp boundary which might be expected of a homogeneous protein. It was recognized, of course, that such a boundary would not in itself establish unequivocally the uniformity of the substance.

We were also interested in comparing zinc-free insulin with zinc insulin and in making comparisons between insulin preparations of varying activity.

By studying the behavior over an extended pH range we hoped to obtain evidence to show whether the changes revealed by the ultracentrifuge at the limits of the pH-stability region proposed by Sjögren and Svedberg (1) are accompanied by corresponding changes in the electrophoretic properties.

Such an investigation as we proposed to make had also within it the possibility of an additional independent examination of the isoelectric point.

Previous electrophoretic studies of insulin have been reported by Howitt and Prideaux (2) and by Wintersteiner and Abramson (3). The method employed in the work of Howitt and Prideaux was based on the direct measurement of the electric mobility of a gold sol coated with an adsorbed film of protein. They arrived at an isoelectric point of 5.4 for the insulin.

\* The insulin used in the present investigation was kindly supplied by Eli Lilly and Company. The author takes this opportunity to express his sincere appreciation for this material.

† Eli Lilly and Company Research Fellow.

Wintersteiner and Abramson made mobility measurements of microscopically visible quartz particles coated with insulin and made a corresponding study of particles of amorphous insulin and suspended insulin crystals. They state that the point of zero mobility for amorphous insulin on quartz lies between pH 5.3 and 5.35. For the suspended insulin crystals they conclude that the point of zero mobility is definitely lower, about pH 5.0.

#### *Methods and Materials*

The new type of Tiselius moving boundary electrophoresis apparatus was used for all of the experiments reported here. The procedure was as specified by Tiselius (4) with electrodes and electrode vessels as suggested by Longworth and MacInnes (5). The temperature of the thermostat was always 0.2°. The position and form of the boundaries were determined by visual observation of the camera image on a ground glass plate, and by the photographic schlieren scanning system of Longworth (6).

The mobilities were calculated by means of the formula

$$u = \frac{\Delta x}{\Delta t} \cdot \frac{q\kappa}{i}$$

where  $\Delta x$  is the distance moved by the descending boundary in time  $\Delta t$ ,  $q$  is the cross-sectional area of the U-tube,  $\kappa$  is the conductance of the protein solution, and  $i$  is the current. The initial boundaries were moved out to a position where they could be clearly seen, and a photograph of the schlieren band was taken. At appropriate intervals during the experiment, a record of the refractive index gradient with reference to the cell position was made by the photographic schlieren scanning system of Longworth (6). The distance,  $\Delta x$ , was taken as the distance from the center of the initial schlieren band to the ordinate which divides the area under this refractive index *versus* distance curve into equal parts. Longworth and MacInnes (7) show that this method of calculation leads to an essentially correct value for the mobility.

*Insulin Preparations Studied*—The following insulin preparations were used in the present investigation: (a) Lilly's crystalline insulin preparations, T-1072 and T-1115 (24 units per mg.); (b) Lilly's amorphous insulin, Iletin W-1002 (21 to 22 units per ing.); (c) insulin crystallized from Iletin W-1002 by Scott's procedure (8); (d) insulin recrystallized from this crystalline insulin

and from the Lilly crystals, T-1072 and T-1115, according to the acetic acid-pyridine-ammonia method as described by du Vigneaud, Miller, and Rodden (9); (e) electrodialed insulin which was made by electro dialysis of either crystalline or recrystallized insulin in 0.001 N HCl until zinc-free. This acid solution was further electro dialyzed with distilled water until the solution showed a trace of turbidity. It was then frozen and the insulin dried *in vacuo*; (f) a solution supplied by Mr. Walden of Eli Lilly and Company, "amorphous precipitate from Zn insulin crystals, C-35." This solution had an activity of 16 units per mg. of solids.

*Preparation of Solutions*—The electrophoresis experiments were carried out between pH 3.3 and 4.2 and between pH 6.8 and 9.6. It was impossible to use the moving boundary method in the intermediate range owing to the insolubility of the insulin at these pH values. For the lower range, acetate and citrate-phosphate buffers were used, while above pH 7, phosphate, phosphate-borate, and borate-carbonate buffers were used.

The insulin solutions were ordinarily prepared for electrophoresis by dialysis to equilibrium with the buffer to be used in the experiment. However, the electro dialyzed and dried insulin preparations contain only a very slight amount of electrolyte and since conductance and pH determinations showed only negligible differences between dialyzed and undialyzed solutions of this material, it was frequently used for electrophoresis without preliminary dialysis.

### Results

*Homogeneity of Insulin Preparations in Acid Solutions*—In the electrophoresis experiments carried out on the acid side of the isoelectric point, insulin gives, in each case, a single boundary which has the appearance and migration behavior expected of a homogeneous protein. Fig. 1 gives the refractive index gradient *versus* distance curve for an experiment with crystalline insulin. All of the other insulin preparations listed above and a number of mixtures of these preparations were investigated and each gives a diagram similar to Fig. 1. No significant differences in mobility could be detected between the various crystalline and amorphous forms of the insulin.

*Effect of Zinc in Acid Solution*—For experiments carried out in phosphate-citrate buffers, the insulin boundary concentration



gradients and the mobilities were found to be uninfluenced by the presence of zinc ions in the buffer. Both crystalline and zinc-free samples were investigated. Each ml. of the buffer contained zinc ion equivalent to 0.5 per cent of the protein concentration used in the experiment. Because of the insolubility of zinc ion, parallel experiments in phosphate buffers were impossible.

*Homogeneity of Insulin Preparations in Alkaline Solutions—*On the alkaline side of the isoelectric point, indication of only one component was obtained in each of the experiments. However, the boundaries were less sharp than at corresponding migration times, buffer salt concentrations, and protein concentrations for

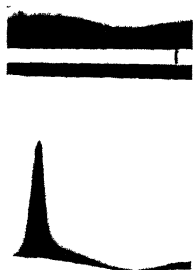


FIG. 1

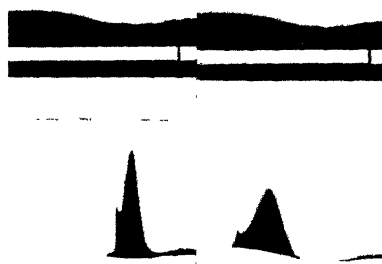


FIG. 2

FIG. 1. Insulin boundary in citrate-phosphate buffer. Concentration of protein 0.2 per cent, pH 3.48, ionic strength of buffer 0.10.

FIG. 2. Insulin boundary in phosphate buffer (a) after 1 hour, 50 minutes migration; (b) after 3 hours, 40 minutes migration. 0.2 per cent protein, pH 7.37, ionic strength of buffer 0.10.

experiments on the acid side of the isoelectric point. Fig. 2 gives a protein concentration gradient *versus* distance curve for a 0.2 per cent solution of crystalline insulin at pH 7.55, while Fig. 3 gives the corresponding curve for a 1.0 per cent solution of the same insulin. Fig. 4 is for a 0.2 per cent electro dialyzed insulin preparation at pH 9.6. The boundaries at pH 9 to 10 are always much sharper than for corresponding experiments at pH 7 to 8. Fig. 2 shows a small but sharp refractive index gradient on the leading side of both ascending and descending boundaries. This gradient always appears in dilute protein solutions in phosphate

buffers. It appears throughout the range of ionic strength used and conductance data for solutions dialyzed for different times indicate that the phenomenon is not due to lack of dialysis equilibrium. The gradient disappears at higher protein concentrations and at higher pH values for all protein concentrations used.

*Comparison of Various Insulin Preparations in Phosphate Buffers*

—Table I shows a representative portion of a great number of data indicating that different insulin preparations give different mobility rates in phosphate buffers. When identical dialysis and experi-

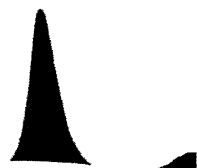


FIG. 3



FIG. 4

FIG. 3. Insulin boundary in phosphate buffer. 1.0 per cent protein, after 3 hours, 40 minutes migration; pH 7.55, ionic strength of buffer 0.10.

FIG. 4. Insulin boundary in carbonate-borate buffer. Protein concentration 0.2 per cent, pH 9.60, ionic strength of buffer 0.10.

mental conditions are used, the crystalline insulin samples show a higher mobility rate than the others; say, under a typical set of conditions about 20 per cent higher than does Lilly's amorphous insulin, W-1002, and 40 per cent higher than the electro dialyzed insulin. However, when the electro dialyzed insulin and crystalline insulin, which have quite different mobility rates under corresponding conditions, are mixed under the same conditions, only one component appears, and this component has a mobility rate near to that of the crystalline insulin. It should be noted that there is only a negligible difference in the mobility of insulin from 0.2 to 1.0 per cent solutions.

*Effect of Method of Solution and Dialysis on Mobility of Insulin in Phosphate Buffers*—The mobility of insulin in phosphate buffers was found to depend upon the method of dissolving the insulin, and upon the time and temperature of dialysis preliminary to the electrophoresis. For crystalline insulin, in a typical experiment at ionic strength of 0.10 and pH 7.56, the following mobilities were obtained: (a)  $5.70 \times 10^{-5}$  when the insulin was dissolved

TABLE I  
*Comparison of Electrophoretic Mobility of Various Insulin Preparations in Phosphate Buffers*

Experiment No.	Insulin used*	Protein concentration	pH	Ionic strength	Mobility	Method of preparation†
		<i>per cent</i>			$\mu \times 10^5$	
189	ED-VI	0.2	7.55	0.10	4.84	(1)
183	T-1072	0.2	7.56	0.10	5.45	(1)
185	"	1.0	7.56	0.10	5.44	(1)
191	"	0.42	7.55	0.10	5.43	(1)
	ED-VI	0.42				
152	W-1002	0.2	7.35	0.02	7.12	(2)
158	"	0.2	7.34	0.02	7.00	(2)
160	ED-VII	0.2	7.34	0.02	6.36	(3)
157	RC-6	0.2	7.44	0.02	8.94	(2)
154	"	0.2	7.48	0.02	8.88	(2)
172	ED-VI	0.2	7.41	0.05	5.20	(3)
142	C-35	0.3	7.42	0.05	6.20	(2)
165	RC-8	0.2	7.44	0.02	6.80	(4)
164	W-1002	0.2	7.34	0.02	7.40	(4)

\* ED refers to electro dialyzed preparations; RC to recrystallized preparations; other numbers to Lilly's preparations (see text).

† (1) dialyzed at room temperature; (2) dialyzed at 0°; (3) undialyzed, dissolved at 0°; (4) dialyzed at room temperature with phosphate buffer, then made acid, and dialyzed again with phosphate buffer at 0°.

directly in the buffer and dialyzed at room temperature; (b)  $5.45 \times 10^{-5}$  when the insulin was dissolved with the minimum amount of acid, returned to pH 7.56, and dialyzed at room temperature; (c)  $5.11 \times 10^{-5}$  when dissolved as in (b) but dialyzed at 0°.

For amorphous insulin, Lilly's iletin W-1002, a higher rate is obtained when it is dissolved in and dialyzed with the phosphate buffer at room temperature than when the dialysis is carried out

at 0°. This insulin contains considerable acid; so a direct comparison with the crystalline insulin is impossible.

As explained previously, the electrodyalyzed insulin could be used for electrophoresis without preliminary dialysis. In the phosphate buffers dialysis at room temperature gives a slight increase in the mobility rate, though mixtures of the dialyzed and undialyzed insulin give no separation.

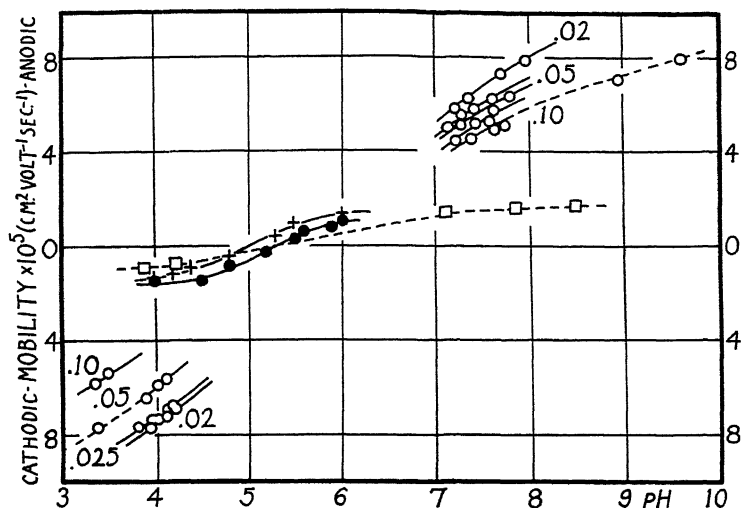


FIG. 5. pH-mobility data for insulin. O present investigation. The broken line indicates different buffer systems at the same ionic strength. □ data of Howitt and Prideaux for insulin-gold sol. ● data of Wintersteiner and Abramson for insulin on quartz. + data of Wintersteiner and Abramson for insulin crystals.

When excess disodium phosphate is used to form a clear solution of iletin, W-1002, a considerably higher mobility is obtained than when the insulin is dissolved directly at a lower pH. This rate is quite difficult to reproduce from experiment to experiment.

It was shown in all cases that differences in pH or conductance of the dialyzed solutions could not be responsible for the results recorded here. The most symmetrical boundaries were obtained upon dialysis for about 24 hours. As the dialysis time at room temperature in phosphate buffers was increased beyond this time,

the boundaries became increasingly diffuse, and showed more and more of the phenomena of reversible boundary spreading.

*pH-Mobility Data*—The mobility data, taken under conditions which permit a pH-mobility plot are included in Fig. 5. The data on the acid side of the isoelectric region from pH 3.8 to 4.3 were obtained in acetate buffers; from pH 3.3 to 3.6 the experiments were carried out in citrate-phosphate buffers. These experiments were, with few exceptions, carried out with the electro-dialyzed insulin and many of them were in undialyzed solution. The data of Fig. 5 from pH 7 to 8 are from experiments with phosphate buffers. The experiment at pH 8.95 was carried out in phosphate-borate, and the one at pH 9.6 was in a borate-carbonate buffer. Electrodialyzed insulin was used in all of these experiments which were either carried out with undialyzed solutions or with solutions which had been dialyzed at 0°. The curve for ionic strength of 0.02 and the upper curve for ionic strengths of 0.05 and 0.10 were determined with electro-dialyzed insulin prepared from the Lilly crystalline insulin. The lower curves for ionic strengths of 0.05 and 0.10 were determined with electro-dialyzed insulin from recrystallized insulin, which in turn had been prepared from T-1072.

#### DISCUSSION

Since the insulin preparations of varying activity all appear to be uniform in electrophoretic properties, it is quite evident that, under the conditions used here, the moving boundary electrophoretic method offers little aid for the preparation and purification of insulin. The fact that different preparations give slightly different behavior in the range pH 7 to 8 does not offer a method of identification of insulin samples. Here all mixtures behave as a single substance and there is no apparent relation between mobility rate and activity. There is no correlation between the mobility differences due to method of solution and dialysis and activity changes during these processes, as assay indicates no significant change in activity during these treatments.

From pH 3.3 to 4.2 the insulin appears to be completely homogeneous. From pH 7 to 8 there is only a slight spread of the electrophoretic properties. At values up to pH 9.6 the boundaries are quite sharp. There is no evidence of abrupt change of the

properties in this entire range. Owing to the insolubility of the preparations studied under the conditions of the experiments from pH 4.5 to 7.0, it is impossible to make any study in the stability region proposed by Sjögren and Svedberg.

Fig. 5 shows in addition to the data of the present work the data of Wintersteiner and Abramson and part of the data of Howitt and Prideaux. Wintersteiner and Abramson assumed that the dissolved insulin would have the same mobility as that precipitated on quartz. Such identity has been demonstrated for egg albumin and serum albumin. Because of the solubility limitations on the moving boundary method, the present work may be compared with that of Wintersteiner and Abramson only around pH 4. The experiments of these authors, carried out in  $m/30$  acetate buffer give a mobility of about 1.5 units at pH 4. The present work includes data in acetate buffers at ionic strengths of 0.02, 0.025, and 0.05, and a mobility extrapolated to the ionic strength of Wintersteiner and Abramson's work is about 7.5. If this mobility of 7.5, taken at  $0.2^\circ$ , is corrected to  $25^\circ$ , it is approximately doubled, which emphasizes the difference.

Accurate extrapolation over the insoluble region is impossible from the present data; so an isoelectric point cannot be accurately determined. However; the data here presented may indicate a higher isoelectric point than that given by Wintersteiner and Abramson and by Howitt and Prideaux, the possible limits being pH 5.3 to 5.9. The curves for the different ionic strengths cross each other in the insoluble region.

The author wishes to express his sincere appreciation to Professor Vincent du Vigneaud for his encouragement and advice during the progress of this work.

#### SUMMARY

1. Crystalline insulin has been studied by the moving boundary electrophoretic technique and found to be homogeneous in the properties revealed by this method.

2. Various other insulin preparations have been studied under the same conditions as the crystalline insulin, and have been found to be indistinguishable from the crystalline insulin. These studies included preparations with activity as low as 16 units per mg.

3. The presence of zinc in the buffers on the acid side of the insoluble region is found to have no effect upon the electrophoresis of insulin.

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## THE LIPIDS OF THE ADRENAL GLAND IN NORMAL AND FASTING RABBITS\*

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Perhaps because micromethods for lipids have only recently been available, the distribution of lipids in the adrenal gland has been almost unexplored. Thus, although a great deal of histological material has been reported, there is by contrast only a single series of total lipid analyses (1). No attempts have been made previously to include phospholipid and neutral fat estimations. The cholesterol content (because cholesterol micromethods have been available for some time) has been reported by several authors (2-7).

### EXPERIMENTAL

#### *Procedure*

Adult albino rabbits, normal and fasting, were chosen as the experimental animals. All rabbits were allowed tap water *ad libitum*. After various fasting periods, the animals were sacrificed painlessly by a blow on the head. The adrenals were quickly removed; the left adrenal glands were reserved for a histological study which will be reported separately by one of us (R. W.) in due course. The right adrenal glands were weighed individually and placed immediately in 95 per cent alcohol.

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They were ground with sand, extracted, and analyzed by standard lipid methods (8-10) (Fig. 1).

### Data

*Adrenal Weight*—The adrenal weights varied widely; the maximum weight was 315, the minimum 53, and the average 93 mg.

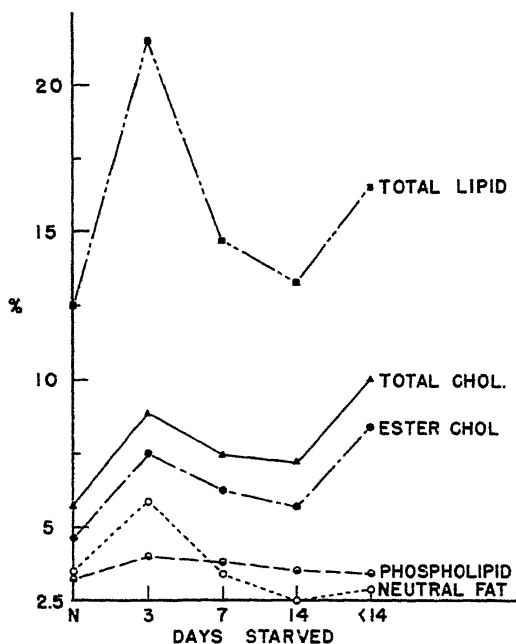


FIG. 1. The average lipid percentages of the moist weight of the adrenal are plotted as ordinates against the number of days the animals were fasted. *N* is the normal unfasted animal. The constancy of the phospholipid is evident, as is the variability of the total lipid. The cholesterol content tends to increase with fasting.

Such widely varying adrenal weights have been reported previously (5-7, 11). There seemed to be no correlation with body weight. The average adrenal weight was about 8 per cent less than normal for the group fasted for 3 days; the averages for the groups fasted 7, 14, and over 14 days were 3, 14, and 11 per cent, respectively, greater than normal.

*Phospholipid*—The phospholipid averages (either in mg. per adrenal or as percentages) for the normal and fasted groups show a remarkable constancy. About 3 to 3.5 mg. of phospholipid are

TABLE I  
*Adrenal Lipid Analyses on Control Rabbits*

Rabbit No.	Initial body weight	Adrenal weight	Phospho- lipid	Neutral fat	Cholesterol		Total lipid
					Total	Ester Total	
	<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>
1	2.75	110	3.49	0.97	7.87	87.2	12.33
2	2.72	98	3.69	2.89	4.77	82.5	11.35
3	2.94	85	2.47	2.81	4.38	84.0	9.66
4	2.51	105	3.79	6.11	5.76	80.5	15.66
5	2.98	77	2.39	4.00	3.54	80.5	9.93
6	2.82	77	2.27	4.86	2.78	78.3	9.91
7	3.05	73	2.55	0.17	6.02	88.5	7.74
8	2.75	75	2.47	4.94	4.24	85.0	11.65
9	2.90	71	2.52	4.67	3.54	84.3	10.73
10	3.00	100	3.07	3.80	3.95	71.8	10.82
11	2.98	73	2.99	2.83	3.93	74.0	9.75
12	3.00	125	3.97	3.03	8.33	78.2	15.33
13	2.55	134	4.97	3.33	10.55	85.0	18.85
14	2.60	100	3.05	2.80	7.22	81.8	13.07
15	2.67	92	2.85	2.25	3.20	76.3	8.30
16	2.64	105	3.04	3.47	8.53	85.1	15.04
17	2.50	100	3.25	2.00	6.18	80.2	11.43
18	2.70	86	2.63	2.91	3.52	77.0	9.06
19	2.58	94	2.86	2.14	5.51	82.3	10.51
20	2.38	83	2.51	2.58	4.96	81.2	10.05
Average.....	2.75	93	3.04	3.13	5.44	81.2	11.45

We are greatly indebted to Miss A. J. Clark of the Department of Bacteriology for a statistical analysis of the chemical data. In each case, she found that the variation between the rabbits in a given group is greater than the variation between the averages of the fasted groups.

present and constitute on the average about 3.5 to 4 per cent of the total moist weight.

*Neutral Fat*—The neutral fat average values tend to rise initially and fall with prolonged fasting. These changes are not great in magnitude; on the average, the increase is of the order of 0.5 mg. and the fall is about 1 mg. Expressed as percentages of

moist adrenal weight, the neutral fat rises from 3.5 to 5.8 per cent in 3 days and then falls after 2 weeks fast to 2.8 per cent.

*Cholesterol*—The adrenal normally contains about 6 per cent of total cholesterol; of this, about 80 per cent is in the esterified form. The total cholesterol shows a steady rise with fasting; the ester percentage remains nearly constant, showing that there is a pro-

TABLE II  
*Adrenal Lipid Analyses on Rabbits Fasted 3 Days*

Rabbit No.	Body weight		Adrenal weight	Phospho-lipid	Neutral fat	Cholesterol		Total lipid
	Initial	Final				Total	Ester Total	
	kg.	kg.	mg.	mg.	mg.	mg.	per cent	mg.
21	2.45	2.05	96	3.78	2.65	6.66	80.4	13.09
22	2.30	2.04	133	5.53		6.26		
23	2.59	2.36	80	3.08	2.28	8.26	87.0	13.62
24	2.40	2.09	81	3.00	1.45	11.25	83.1	15.70
25	2.53	2.35	135	5.16		9.88		
26	2.60	2.22	134	5.20	7.58	9.58	95.3	22.36
27	2.31	2.15	78	3.06		6.54		
28	2.14	1.75	91	3.53	4.17	6.46	86.3	14.16
29	2.61	2.29	82	3.35	2.91	7.44	86.0	13.70
30	2.61	2.34	79	3.48	1.80	6.72	63.3	12.00
31	2.45	2.08		3.28	4.48	6.22	80.0	13.98
32	2.58	2.20		3.40	2.75	7.30	95.0	13.45
33	2.58	2.18		2.47	2.89	4.64	91.6	10.00
34	2.24	2.08		2.55	3.00	2.85	82.1	8.40
35	2.35	2.11	74	2.92	4.82	7.50	87.9	15.24
36	2.25	2.10	53	2.67	4.47	6.29	86.5	13.43
37	2.09	1.87	75	3.10	4.39	5.70	89.3	13.19
38	2.25	2.02	47	1.70		1.86		
39	2.22	2.00	73	2.82	4.86	8.34	83.2	16.02
40	2.30	2.02	71	2.98	4.22	6.08	76.6	13.28
Average . . .	2.39	2.12	86	3.35	3.67	6.79	84.6	13.85

portionate increase in both fractions. The total cholesterol rises from 5.4 mg. initially to 9.9 mg. after 2 weeks fasting.

The amount of cholesterol per adrenal gland in normal rabbits has been reported as 3 to 5 mg. (7) and 4 to 11 mg. (6). In the present work, the average total cholesterol percentages for the normal and fasted groups (Tables I to V) range from about 5.5

to 10; these values are within the previously given limits. The minimum cholesterol found was 2.0 mg. (1.5 per cent); the maximum content was 17 mg. (16.0 per cent). Several authors have reported decreases in cholesterol following certain experimental procedures, (a) after unilateral adrenalectomy plus adrenal insufficiency or plus infection (7), (b) after a single intravenous

TABLE III  
*Adrenal Lipid Analyses on Rabbits Fasted 7 Days*

Rabbit No.	Body weight		Adrenal weight	Phospho-lipid	Neutral fat	Cholesterol		Total lipid
	Initial	Final				Total	Ester Total	
	kg.	kg.	mg.	mg.	mg.	mg.	per cent	mg.
41	2.32	1.80	117	4.38	4.64	9.12	83.8	18.14
42	2.37	2.00	111	3.73	5.08	7.89	81.8	16.70
43	2.42	1.95	66	2.61	3.70	3.02	80.1	9.33
44	2.44	2.10	106	3.76	3.61	6.69	78.2	14.06
45	2.75	2.29	112	4.27	3.11	7.79	84.5	15.17
46	2.68	2.29	124	4.57	2.89	6.18	76.6	13.64
47	2.55	2.08	75					
48	2.36	1.95	94	3.50	3.42	4.62	85.7	11.54
49	2.54	2.05	74	3.20	2.85	6.23	93.5	12.28
50	2.45	2.17	80	3.33	2.76	6.40	76.6	12.49
51	2.35	2.02	93	3.65	2.39	7.56	86.8	13.60
52	2.53	2.20	100	3.63	4.06	10.1	96.5	17.79
53	2.86	2.28	93	3.58	1.60	9.94	86.5	15.12
54	2.42	2.07	152	5.92	3.28	7.36	88.3	16.56
55	2.42	1.95	82	3.12	3.39	3.65	83.5	10.16
56	2.24	1.75	86	3.30	2.50	4.86	80.7	10.48
57	2.35	1.97	54	2.23	1.19	4.55	89.4	7.97
58	2.16	1.57	111	3.88	4.75	11.40	73.9	20.03
59	2.20	1.63	79	3.15	2.44	6.76	89.9	12.35
60	2.75	2.22	115	3.90	4.83	13.45	72.9	22.18
Average . . .	2.46	2.02	96	3.66	3.29	7.23	83.6	14.19

injection of cholesterol (6), and (c) after a single dose of insulin (5). It is noteworthy that an increase in cholesterol content accompanies fasting.

*Total Lipid*—The total lipid is normally about 12 per cent of the fresh adrenal. After 3 days fast, the total lipid percentage has risen to 21; the values fall thereafter and remain at a level of about

TABLE IV  
*Adrenal Lipid Analyses on Rabbits Fasted 14 Days*

Rabbit No.	Body weight		Adrenal weight	Phospho-lipid	Neutral fat	Cholesterol		Total lipid
	Initial	Final				Total	Ester Total	
	kg.	kg.	mg.	mg.	mg.	mg.	per cent	mg.
61	2.57	1.37	73	2.79	3.77	1.49	67.7	8.05
62	2.55	1.82	183	5.99	1.47	13.83	78.9	21.29
63	2.50	1.46	92	3.35	3.72	5.84	70.0	12.91
64	2.64	1.61	80	2.83	4.00	6.52	70.8	13.35
65	2.61	1.84	315	10.9	3.11	23.00	71.2	27.20
66	2.51	1.42	75	2.78	2.75	3.45	76.7	8.98
67	2.30	1.70	73	2.75	2.14	6.55	91.7	11.44
68	2.34	1.45	85	2.65	0.78	6.79	78.5	10.22
69	2.75	1.77	91	3.59	2.80	5.35	82.5	11.74
70	2.65	1.52	99	3.78	2.78	3.88	70.2	10.44
71	2.84	2.00	89	3.14	1.78	8.60	76.2	13.52
72	2.23	1.54	66	2.36	1.42	6.02	82.3	9.80
73	2.71	1.78	98	3.30	2.50	7.47	80.3	13.27
74	2.84	1.92	110	3.72	1.64	7.42	77.0	12.78
75	2.55	1.66	107	3.69	1.22	9.35	82.7	14.26
76	2.69	1.75	150	4.17	0.04	13.17	84.0	17.38
77	2.30	1.71	116	3.56	0.24	11.45	83.8	15.25
78	2.65	1.82	62	2.34	2.75	4.07	86.5	9.16
79	2.58	1.89	86	3.28	2.28	5.55	96.4	11.11
80	2.22	1.49	84	3.32	3.39	6.29	78.7	13.00
Average . . .	2.55	1.67	107	3.72	2.23	7.80	79.3	13.25

TABLE V  
*Adrenal Lipid Analyses on Rabbits Fasted More Than 14 Days*

Rabbit No.	Body weight		Adrenal weight	Phospho-lipid	Neutral fat	Cholesterol		Total lipid
	Initial	Final				Total	Ester Total	
	kg.	kg.	mg.	mg.	mg.	mg.	per cent	mg.
82	2.15	1.20	85	2.88	3.11	9.70	89.3	15.69
83	2.23	1.13	132	3.52	6.53	8.96	86.2	19.01
84	2.28	1.50	73	2.47	3.83	9.54	87.1	15.84
85	2.37	1.25	84	3.13	0.89	10.16	74.9	14.18
86	2.30	1.30	58	2.52	3.47	4.08	90.2	10.07
90	2.54	1.50	97	4.45	2.78	3.84	83.6	11.07
92	2.57	1.35	211	5.47	0.0	13.89	79.7	19.36
94	1.96	1.20	97	3.72	1.80	8.68	83.4	14.20
98	2.30	1.25	94	3.08	0.0	16.02	82.9	19.10
100	2.33	1.25	99	3.15	3.23	13.89	78.3	20.27
Average . . .	2.30	1.29	103	3.44	2.56	9.88	83.6	15.88

14 per cent. This rise is due to an increase in the absolute amounts of neutral fat and cholesterol, since by the 3rd day there is only a small loss in adrenal weight with fasting and thereafter the adrenal weights are greater than normal. Addis *et al.* (12) have noted in rats that no protein is taken from the adrenal in fasting. The only total lipid data in the available literature are those of Grundland and Bulliard (1) who applied the method of Kumagawa to ten adrenals. They found 22 to 49 mg. of total lipid per adrenal, which constituted 73 to 80 per cent of the adrenal dry weight. Such values indicate approximately 20 per cent total lipid on a moist weight basis; this is considerably more than the 12 per cent reported here for normal fresh adrenals.

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## THE CAROTENOID AND PROVITAMIN A CONTENT OF THE WATERMELON

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10 years ago lycopene,  $C_{40}H_{56}$ , and carotene,  $C_{40}H_{56}$ , were isolated from the pulp of the European watermelon (*Citrullus vulgaris*, Schrad. = *Cucumis citrullus*, L.) by Zechmeister and Tuzson (1), and it was shown that the chief pigment, lycopene, is responsible for the red color. As the chromatographic method was not available at that time, no precise information as to the composition of the pigment was obtained. In some new experiments described below we have carried out a quantitative analysis of the components and have estimated the provitamin A content of the California watermelon. 1 kilo of the pulp examined contained 1.0 mg. of a complicated xanthophyll mixture, 6.1 mg. of lycopene, 0.06 mg. of  $\gamma$ -carotene, 0.16 mg. of unknown carotenoids (located in the column between  $\gamma$ - and  $\beta$ -carotene), 0.46 mg. of  $\beta$ -carotene, 0.01 mg. of  $\alpha$ -carotene. The figures include the fractions of lycopene, and  $\gamma$ - and  $\beta$ -carotene which underwent isomerization during the experimental procedure (2).

The colorimetric value of the total extract of 1 kilo of pulp corresponded to 7 to 8 mg. of "lycopene"; some samples were, however, considerably richer in pigment. Our material, picked in California in September, contained, according to the above figures, 0.5 mg. of provitamin A in 1 kilo of pulp, or about one-fifth to one-sixth of the daily  $\beta$ -carotene requirement of an adult person.

It is interesting to note that a considerable number of yellow and pink unidentified oxygen-containing carotenoids were found in minute quantities; *i.e.*, to the extent of about 0.01 mg. per kilo of pulp. Even with the use of chromatography 1000 or more kilos of melon would be needed for a satisfactory study of these

\* Contribution No. 808.



pigments. One of them is spectroscopically identical with torulene, detected by Lederer in red yeast (3).

#### EXPERIMENTAL

##### *Methods*

The two parts of the chromatographic tubes were connected by a ground glass joint and the lower one was equipped with a perforated glass filter plate, as previously described (4).<sup>1</sup> All spectra were determined in an "evaluating grating" spectroscope as devised by Loewe and Schumm and manufactured by Zeiss (Jena). In accordance with a suggestion by Emerson and Fox (5), Jena colored optical filters No. BG-7 were adopted as light filters. For the quantitative estimation of the carotenoids a Pulfrich gradation photometer (Zeiss) was used, with light filter No. S-45 or S-47; the new photometric values given by Cholnoky (6) were used as a standard. Since no such data are available for  $\gamma$ -carotene and for torulene, an average of the colorimetric values of  $\beta$ -carotene and of lycopene in petroleum ether<sup>2</sup> is the basis of the calculations for the  $\gamma$  compound, and Cholnoky's capsorubin values are used for torulene.

##### *Color Analysis of Pulp*

The pulp of a 7 kilo melon (Klondike, 4.3 kilos without seeds) was mashed in a meat chopper and pressed in a hand press. The main pigment content was in the pulp; the small amount in the juice was in the floating red particles. After the addition of 0.05 volume of acetone, the liquid was kept in a narrow bottle overnight. A fine red material settled from which the clear liquid was cautiously decanted. The sediment was filtered off and the conic filter paper containing the insoluble material was dried at 40°. The pressed pulp was kept in methanol overnight and again pressed out. The slightly colored methyl alcohol was then added to the acetone-containing liquid mentioned above, and all the pigment was extracted by repeated shaking with a 1:1 mixture of benzene and petroleum ether. After evaporation the residue was dissolved in ether (Fraction I).

<sup>1</sup> This device is now manufactured by the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

<sup>2</sup> The petroleum ether referred to throughout this paper is that having a boiling point of 60-70°.

The pressed material was dried at 40° (16 gm.) and combined with the dry sediment and this brownish red, hard material ground in a mill and ether percolated through it. The extraction was completed by using a smaller quantity of carbon disulfide which was collected separately and evaporated to dryness, and the residue dissolved in the ether extract and combined with Fraction I. The solution (450 cc.), containing practically all of the pigment present in the pulp, was saponified with 50 cc. of concentrated methanolic KOH overnight. After the addition of approximately 1 volume of water, the alkaline layer was discarded, the red solution washed three times with water, and the saponification repeated. The pigment solution was washed until free from alkali, dried over sodium sulfate, and evaporated to dryness under diminished pressure in a slow stream of CO<sub>2</sub>.

The dark residue was dissolved in 300 cc. of petroleum ether. The pigment content of this solution corresponded to 35 mg. of lycopene. It was chromatographed on calcium hydroxide (Shell Brand, chemical hydrated lime, 98.5 per cent; tube, 30 × 5.5 cm.) and developed first with petroleum ether, and later with benzene, until the following picture was obtained (the figures denote the height of the respective zones).

*Fraction A*—3 mm., brownish red; 2 mm., pale intermediate zone; 2 mm., red; 62 mm., bright red, the lower part stronger (lycopene); 11 mm., orange (neolycopene); 4 mm., light orange by-product of the isomerization.

*Fraction B*—10 mm., colorless; 15 mm., pale blurred orange-red; 18 mm., colorless; 20 mm., orange ( $\beta$ -carotene).

The column was cut into two parts; Fraction A was eluted with an alcohol-benzene mixture (1:4), and Fraction B with alcohol and petroleum ether (1:1). After the alcohol had been washed out of both solutions with water, Fraction A was dried, evaporated under diminished pressure, dissolved in petroleum ether, and chromatographed on calcium carbonate (Merck's heavy powder; tube as above). Fraction B was dried and rechromatographed on calcium hydroxide (Merck; tube 23 × 3 cm.). Both columns were developed with petroleum ether.

*Chromatogram A*—Xanthophylls: 5 mm., many thin yellow lines; 5 mm., several red and yellow lines; 2 mm., almost colorless; 5 mm., several pink and yellow lines.

Hydrocarbons: 60 mm., almost colorless; 95 mm., dark orange (lycopene); 4 mm., orange (neolycopene).

The lycopene and neolycopene layers contained 26.2 mg. of pigment. Maxima of lycopene, 547, 507.5, 475.5  $m\mu$ ; of neolycopene, 536, 498, 466  $m\mu$  (in carbon disulfide).

It was impossible to separate the individual xanthophylls, the total color intensity of which corresponded to 4.3 mg. of "lutein". This xanthophyll fraction gave, when rechromatographed on  $\text{CaCO}_3$ , at least twenty to twenty-five colored zones, some of which had been formed by isomerization. All were poorly separated. The majority of the yellow lines were located near the top, while in the next lower section pink and yellow lines were preponderant. The spectra of all these zones, taken in carbon disulfide, vary between 497.5, 466.5, 438  $m\mu$  and 568.5, 528, 493  $m\mu$ . The carotenoid showing the highest maxima, of which a few crystals were obtained, is spectroscopically identical with torulene; maxima in petroleum ether, 523.5, 491, 455.5  $m\mu$ ; in benzene, 539, 496, 459  $m\mu$ . The first bands are sharply bordered but narrow; the second ones appear broader and stronger. We believe that the quantity of this pigment is of the order of magnitude of 0.01 mg. in 4.3 kilos of the pulp.

*Chromatogram B (Spectra in  $\text{CS}_2$ )*—30 mm., colorless; 10 mm., dark orange ( $\gamma$ -carotene, 0.14 mg., 532.5, 494.5, 461.5, 430.5  $m\mu$ ); 3 mm., colorless; 15 mm., yellow (neo- $\gamma$ -carotene,<sup>3</sup> 0.15 mg., (527), 492, 458.5  $m\mu$ ); 15 mm., lemon-yellow (unknown, 0.07 mg., (525), 490.5, 458.5, 430  $m\mu$ ); 8 mm., colorless; 20 mm., orange (unknown, 0.62 mg., 512.5, 479.5, 452  $m\mu$ ); 3 mm., colorless; 15 mm., dark orange ( $\beta$ -carotene, 1.5 mg., 520, 484, 454  $m\mu$ ); 1 mm., colorless; 13 mm., light orange (neo- $\beta$ -carotene,<sup>4</sup> 0.5 mg., 513, 479  $m\mu$ ); 10 mm., blurred yellow (unknown, 0.04 mg.;  $\alpha$ -carotene?).

#### *Isolation of Lycopene and of $\beta$ -Carotene*

The preparation of the material and the extraction were carried out as described above, with the exception that the sediment was separated in the centrifuge. 83 kilos of watermelon (variety Rattlesnake) gave about 50 kilos of pulp, 490 gm. of dried brownish red powder, and finally 500 cc. of a dark petroleum ether solution which contained the total saponified pigment. Two chromatographic tubes (30  $\times$  7.5 cm.) were necessary. The

<sup>3</sup> This layer was identified as neo- $\gamma$ -carotene by partial conversion into  $\gamma$ -carotene on standing. We intend to describe the isomerization of this hydrocarbon later.

<sup>4</sup> Termed also pseudo- $\alpha$ -carotene.

sequence of the zones was like that of the first chromatogram described above, the separation, however, being much less clear, owing to the comparatively higher amount of pigment per unit weight of adsorbent.

*$\beta$ -Carotene*—The layer was cut out, eluted with an alcohol and petroleum ether mixture, rechromatographed, transferred into petroleum ether, and, after evaporation of the latter, crystallized from benzene after addition of methanol. The yield was 19 mg. of glittering plates. Maxima in carbon disulfide, 520, 485  $m\mu$ ; in petroleum ether, 485, 452.5  $m\mu$ . No separation from added  $\beta$ -carotene (from carrots) was obtained in the column. The mother liquor contained a considerable quantity of crystallizable sterols and other colorless substances, as well as 1.5 mg. of carotene.

*Lycopene*—The lycopene and the neolycopene layers were cut out together with all the layers above  $\beta$ -carotene. This section was rechromatographed on calcium carbonate (precipitated, McKesson) in seven portions; each time a  $30 \times 5.5$  cm. tube was used and the column washed with large quantities of petroleum ether, whereupon a sharp separation of the top layers from the lycopene layer resulted. The latter was cut out, eluted with alcohol and petroleum ether (1:1), and chromatographed on  $\text{CaCO}_3$ ; it now showed only negligible zones above the lycopene. After elution the main pigment was transferred into petroleum ether which was dried and evaporated. The residue was dissolved in the smallest possible amount of carbon disulfide, diluted with petroleum ether, and the pigment crystallized out by addition of absolute ethyl alcohol. The yield was 217 mg. of long red needles. From another variety of watermelon (Klondike) the corresponding yield was 188 mg. Maxima in carbon disulfide, 547, 507.5, 475.5  $m\mu$ ; in petroleum ether, 503.5, 473.5, 445  $m\mu$ . No separation from tomato lycopene could be obtained in the Tswett column.

#### SUMMARY

The carotenoids of watermelon pulp were chromatographed and estimated. The provitamin A content was about 0.5 mg. of  $\beta$ -carotene per kilo of pulp and is of the order of magnitude found by Munsell (7) in feeding experiments with rats.

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## FRACTIONAL DISTILLATION OF UNSATURATED FATTY ACIDS

### I. THE EFFECT OF VACUUM DISTILLATION ON THE ABSORPTION SPECTRA OF POLYETHENOID ESTERS FROM COD LIVER OIL\*

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The analysis of fatty acids of animal and vegetable origin by vacuum distillation of the methyl esters through packed fractionating columns (1) is based on the assumption that the original constituents undergo no chemical changes during the distillation process. With saturated and certain unsaturated fats containing one to three double bonds in the hydrocarbon chain, such an assumption is probably valid. In the case of marine and other oils containing highly unsaturated acids, however, it is not improbable that the distillation process is accompanied by more or less pronounced chemical changes because of the tendency of these acids to conjugate and polymerize when exposed to relatively high temperatures for prolonged periods of time.

Farmer and Van den Heuvel (2) have emphasized the fact that highly unsaturated fatty acids, such as those found in cod liver and similar fish liver oils, are very sensitive to heat. Thus, it is stated that below 200° these acids undergo changes, which "are in part polymerisations but appear to some extent to be isomerisations." Evidence for this conclusion is based mainly on the finding of exalted molecular refraction in highly unsaturated distillates obtained by the use of a vacuum fractionating column. Abnormal molecular refraction is also noted in all the supposedly homogene-

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ous tri- and polyolefinic acids prepared from fish oils by other investigators using distillation methods. Since the original oils and their undistilled fatty acids exhibit normal refractivity, this optical anomaly is due presumably to conjugation induced by heat.

A recent corroboration of this conjugation hypothesis is found in a paper by Shinowara and Brown (3) in which it is noted that distilled highly purified methyl arachidonate possessed diene values indicative of "5 per cent conjugation."

In an attempt to isolate some of the most highly unsaturated fatty acids of cod liver oil without subjecting them to any possible effects of high temperatures, Farmer and Van den Heuvel resorted to molecular distillation in a "continuous still." The temperature was maintained below 110° and the time of contact between the heating element and the ester film was reduced to 60 seconds. In this way, a  $C_{22}$  ester fraction was obtained, which was found to be pure docosahexaenoic acid,  $C_{22}H_{32}O_2$ . No five double bond  $C_{22}$  acid, comparable with the clupanodonic acid of Tsujimoto and Kimura (4), could be isolated. These investigators thus concluded that the five double bond acid was not present originally in the oil, but was probably formed by thermal decomposition of a six double bond (docosahexaenoic) acid during the distillation process.

In order to follow the extent of chemical changes occurring during careful fractional distillation of highly unsaturated fatty acid esters, absorption spectra are more useful than molecular refractions, since the former not only are more sensitive, but may also, through characteristic absorption curves, give some indication of the nature of any rearrangements occurring. Therefore, in this study we have determined the absorption curves as well as the customary iodine numbers and saponification equivalents of a series of ester fractions resulting from fractional distillation, using a packed column, of highly unsaturated cod liver oil esters prepared by crystallization at low temperature of the free fatty acids and their subsequent esterification. Since previous work in this laboratory has indicated that untreated cod liver oil exhibits some spectroscopic absorption, the present investigation was undertaken to determine (1) whether absorption (or refraction) in high boiling distillates is due merely to fractionation of the absorptive components or to chemical changes occurring during distillation,

and (2) if chemical changes occur, whether they are sufficient to invalidate the use of a vacuum fractionating column for analytical and isolation work. Any appreciable isomerization of polyethenoid acids to conjugated forms, for example, is of extreme analytical importance, since some of the constants used in the

TABLE I  
*Chemical Constants of Methyl Ester Fractions of Cod Liver Oil*

Sample No.	B.p. at 1 mm. pressure	Weight of fraction	Iodine* No. (Wijs-Hg acetate method (7))	Saponification* equivalent	Total heating time	Liberation of iodine from Wijs solution (8, 9)
	°C.	gm.			min.	
Original cod liver oil		110.0	183.8		None	None
1	133-136	4.08	67.5	255.8	65	"
2	137-139	4.78	76.7	256.2	95	"
3	139-144	5.41	87.6	260.8	120	"
4	145-146	4.44	97.5	264.6	137	"
5	146-149	3.35	101.7	267.4	164	"
6	150-154	11.06	116.0	280.5	200	"
7†	155-156	8.94	118.0	283.8	338†	"
8	157-159	6.81	130.8	288.9	374	"
9	160-165	4.06	153.7	295.6	386	"
10	166-169	6.22	186.7	300.2	401	"
11	170	3.04	204.1	303.1	421	"
12	171-175	15.11	223.5	306.4	465	Slight
13	178-181	6.20	240.0	316.9	476	"
14	184-190	3.32	251.9	320.6	483	"
15	191	9.73	254.5	333.3	493	"
16	Residue	13.0	243.8	370.3		Pronounced

\* These determinations were made with the assistance of Mr. Vernon Wystrach.

† Distillation was interrupted for 52 minutes before this fraction was collected. The bath heat was shut off, and no distillate removed during this time.

calculation of the composition of the fat, specifically iodine and thiocyanogen values, will then give only misleading and incorrect results (5). Furthermore, the extent of polymerization or conjugation must be known at least approximately in order to decide whether or not high boiling ester fractions are sufficiently representative of the original sample to be used for isolation and structure studies.



## EXPERIMENTAL

*Preparation of Unsaturated Methyl Esters*—A 750 gm. sample of cod liver oil was saponified for 15 minutes with 20 per cent alcoholic KOH. After acidification, the fatty acids were taken up in ligroin (b.p. 30–60°), freed of mineral acid by washing with water,

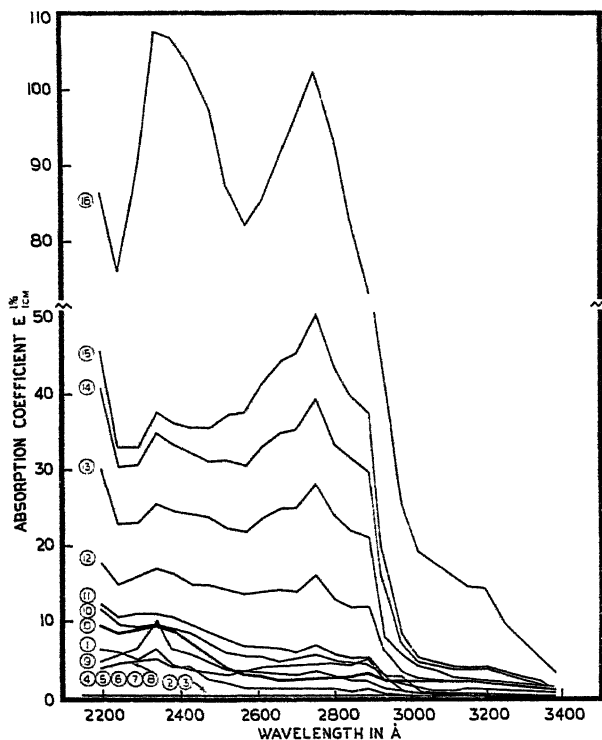


FIG. 1. Absorption curves of fractions obtained by vacuum distillation of cod liver oil esters. The figures on the curves represent the sample numbers.

and dried with sodium sulfate. The fatty acids remaining after evaporation of the ligroin *in vacuo* weighed 619 gm. and had an iodine number of 175. These acids were taken up in 1 liter of acetone, cooled to  $-10^{\circ}$ , and the solution filtered. Three more repetitions of the cooling process yielded a filtrate containing

fatty acids with an iodine number of 193 and a total of four precipitates containing saturated and some unsaturated fatty acids (iodine numbers 112 to 149). The acids in the filtrate were converted to their methyl esters by refluxing with 700 ml. of 5 per cent methyl alcoholic sulfuric acid for 2.5 hours. A 110 gm. sample of the isolated esters (iodine number 183.8) was used for distillation.

*Distillation of Methyl Esters*—The fractionating column used in these experiments is similar in form to that described by Longenecker (6). The distillation column, 60 cm. in length and 17 mm. in diameter, was packed with 3 mm. single turn glass helices. Distillations were made at a pressure of 1 mm. of Hg as measured by placing a manometer between the still head and vacuum pump. The temperature of the heating bath was approximately 10° higher than that of the fraction distilling, and the temperature in the column was 20° higher than that of the bath. Distillations were performed at an average rate of approximately 14 gm. per hour.

Table I summarizes the distillation data obtained.

*Absorption Spectra of Ester Fractions*—All absorption spectra were obtained by the use of a photoelectric spectrophotometer described elsewhere (10). Fig. 1 summarizes the data obtained.

#### DISCUSSION

The absorption curves reported in Fig. 1 show that during a vacuum fractional distillation of unsaturated cod liver oil esters two processes take place simultaneously. There is some fractionation of the initially absorptive components of the esters, as indicated by the decreased absorption of the first few distillates compared to that of the undistilled esters, and coincident with this there is a gradual isomerization process which produces more absorptive material as the distillation time increases. Thus, a summation of the "absorption indices" (absorption coefficient  $\times$  weight of fraction) at 2350 and 2700 Å. of all the distillates plus the residue shows that the total absorption at 2350 Å. is increased 3-fold by distillation, and the absorption at 2700 Å. is increased 8-fold. The wave-lengths 2350 and 2700 Å. are selected for consideration because absorption maxima at these points are noted in all the later distillates and, therefore, constitute the most easily

measured indication of the changes taking place during distillation.

It must be remembered that while absorption is increased, the absorptive material concentrates in the residue, leaving the distillates comparatively unaffected. Thus, the absorption of the undistilled esters is not equaled in any fraction until Sample 9 is reached; and although Sample 12 is sufficiently changed to enable it to liberate iodine from iodine monochloride (Wijs solution), a phenomenon usually exhibited only by substances containing conjugated double bonds, the following spectroscopic and chemical considerations indicate that the fraction is still sufficiently unaltered to make it suitable for analytical and isolation work.

Any attempt to account quantitatively for the absorption data as conjugation must admittedly yield only approximate results, since the absorption coefficients for completely conjugated or otherwise isomerized cod liver oil esters are not available.<sup>1</sup> However, if one assumes that all absorption at 2350 Å. is due to two conjugated double bonds such as are found in 10,12-linoleic acid, and that all absorption at 2700 Å. is due to three conjugated double bonds such as are found in the eleostearic acids, then it is possible to make a rough calculation of the extent of conjugation in the distilled fractions from the absorption coefficients observed. On this basis, the highest boiling fraction ( $E_{1\text{cm.}}^{1\%} = 50.6$  at 2700 Å. and 37.8 at 2350 Å.) contains 2 to 3 per cent of triply conjugated linkages and even less doubly conjugated linkages.

This is in complete agreement with chemical findings, since one of the most absorptive fractions, Sample 13, exhibited the same iodine number, 240, with both the Wijs and Hanus solutions, although the latter reagent is known to yield much higher values than the Wijs solution when conjugated double bonds are present.

It is noteworthy that several investigators have obtained absorption maxima at 2300 and 2700 Å. by prolonged saponification of cod liver oil. The highest absorption coefficients have been reported by Edisbury *et al.* (11);  $E_{1\text{cm.}}^{1\%} = 200$  at 2300 Å. for total cod liver oil acids and an  $E_{1\text{cm.}}^{1\%} = 1000$  at 2700 Å. for liquid C<sub>20</sub> cod liver oil acids. Edisbury attributes the absorption to cyclization on the basis of his spectrographic identification of aromatic nuclei in the selenium dehydrogenation products from

<sup>1</sup> This aspect of the problem is now being investigated.

absorptive material, as well as the non-specific type of absorption curves observed. Other investigators (12), however, believe that some form of double bond isomerism such as a change in position in the carbon chain is responsible, and, recently, conjugation of vegetable oils has been produced by prolonged saponification at high temperatures (13). Nevertheless, one cannot compare changes produced by heat alone to those produced by alkali and heat without first proving the identity of the results; so these experimental values for cod liver oil cannot be used for any calculation of per cent of rearrangement or conjugation.

Our experimental results show that very little rearrangement has occurred during the fairly prolonged fractional distillation. This is corroborated by another experiment in which 50 gm. of highly unsaturated methyl esters of cod liver oil (iodine number 262), prepared by fractional crystallization at low temperature and subsequent esterification, were fractionally distilled during a period of 200 minutes. The highest boiling fraction, iodine number 365.5, had an  $E_{1\text{cm}}^{1\%} = 43$  at 2700 Å., equivalent to only 2.0 per cent conjugation. Unless some loss of double bonds occurs, as suggested by Farmer and Van den Heuvel in order to account for their ability to secure by molecular distillation only a six and not a five double bond  $C_{22}$  acid comparable with the clupanodonic acid of Tsujimoto, it is evident that the amount of rearrangements produced in distillates during vacuum distillation is insufficient to interfere with analytical calculations and isolation work.

#### SUMMARY

Spectroscopic and chemical evidence indicates that distillates obtained by vacuum fractional distillation of methyl esters of highly unsaturated fatty acids are sufficiently representative of the original material to be used in isolation and structure work. Analytical applications of the process are limited by the concentration of isomerized material in the residue.

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## A METHOD FOR THE DETERMINATION IN VITRO OF RIBOFLAVIN IN TISSUES

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The problem here undertaken was the development of a method for the determination of riboflavin *in vitro*, with special reference to its applicability to animal tissues. For many years feeding methods have given the most dependable results but more accurate methods which would require less time and material have been sought. Recently several *in vitro* methods for the determination of riboflavin have been proposed and the adaptation of one of these offered a possible solution.

Among those who have used *in vitro* methods, Warburg and Christian (31), Kuhn and coworkers (20), and later Schormüller (25) estimated the amount of the photochemical decomposition product, lumiflavin, with a stage photometer. Koschara (18) and Gourévitch (13) used the same instrument but measured the riboflavin concentrations directly after the extracts had been subjected to different purification processes. Charite and Khaustov (6) and Murthy (21) employed colorimetry with potassium dichromate or riboflavin standards. Visual comparison of the fluorescence of unknown and standard riboflavin solutions has been the basis of many determinations (von Euler and Adler (10), Supplee, Ansbacher, and Bender (27), Whitnah, Kunerth, and Kramer (33), and Weisberg and Levin (32) who differed in the fact that they used fluorescein standards). Other determinations have been made with photoelectric cells. Josephy (16) measured the intensity of fluorescence of lumiflavin; Cohen (7) that of riboflavin; Sullivan (26) the light absorption of riboflavin; Hand (14) the fluorescence in comparison with that of uranium glass standards; Hodson and Norris (15) determined riboflavin indirectly by photometric estimations of the fluorescence of solutions before

and after reduction with sodium hydrosulfite; and Kahler and Davis (17) measured the total fluorescence, then eliminated the riboflavin by increasing the alkalinity, and determined the "interfering" fluorescence.

Several of these methods have been applied to the quantitative determination of riboflavin in the organs of rats (12, 17, 19, 24, 29, 30) but simultaneous quantitative determinations by the feeding method have not been made. There is evidence in the literature on this subject indicating the need of modifying the existing *in vitro* methods and of conducting parallel determinations by methods of the two types. Supplee, Bender, and Jensen (28) reported fluorimetric and biological assays of miscellaneous food products with a range in percentage correlation from 95 to 42, neither method giving consistently high or low results. Ellinger (9) has criticized the application of *in vitro* methods to plant and animal products, emphasizing the errors due to incomplete extraction, loss in purification, and difficulties in final estimation, usually resulting in low values. Thus the need exists for further investigation of *in vitro* methods for the determination of riboflavin in tissues.

#### EXPERIMENTAL

*Feeding Method*—The feeding method employed was a modification of the Bourquin-Sherman quantitative determination of vitamin G (5). This has been shown to give a quantitative measurement of riboflavin (1-4). Young albino rats, weighing between 45 and 55 gm., whose mothers were reared on diets containing adequate but not excessive amounts of riboflavin, were separated at 28 or 29 days of age, placed in individual cages, and given riboflavin-deficient Diet 581 and distilled water *ad libitum*. The diet consisted of casein (Labco vitamin-free) 18 per cent, Osborne and Mendel salt mixture (22) 4, butter fat 8, cod liver oil 2, Labco rice polish concentrate 2, and corn-starch 66. After 1 week the animals were harnessed according to the technique of Page (23) to prevent coprophagy. The rats were weighed once weekly for 2 weeks and then daily until their weights were constant and they were thus judged to be depleted. The animals were then divided into three groups, positive controls that received known amounts of a solution of crystalline riboflavin six

times a week, test animals that were given the tissue supplements three times a week, and negative controls that received only the basal riboflavin-deficient diet. The division was made so that there was an equal sex distribution and the same total weight in the first two groups. The test period was 4 weeks long and the animals were weighed weekly.

*History and Preparation of Tissues Used As Supplements*—The tissues of healthy adult rats reared on Diets 16, 473, 463, and 453

TABLE I  
*Composition of Diets (Measured in Gm.)*

Constituents	Diet 473	Diet 463	Diet 453
Skim milk powder .....	160	160	160
Casein.....	60	60*	60†
Osborne and Mendel salt mixture (22).....	20	20	20
Butter fat.....	80	80	80
Lactose.....	80	80	80
Cod liver oil.....	20	20	20
Whole wheat‡ .....	556	556	556
Dicalcium phosphate.. ..	4	4	4
Agar.....	20	20	20
Diet 16			
Whole wheat.....	1000		
“ milk powder.....	200		
Sodium chloride.....	20		

\* 13 mg. (13 cc.) of 1E lactoflavin (The Borden Company) dried on casein.

† 26 mg. (26 cc.) of 1E lactoflavin (The Borden Company) dried on casein.

‡ 840 gm. of whole wheat as alcohol extract dried on whole wheat.

were used. The riboflavin contents of these diets determined by the above method are 4.0, 5.1, 15.0, and 23.0  $\gamma$  per gm. respectively. Detailed information concerning the composition of these diets is given in Table I.

The rats were stunned and decapitated in order to free the tissues of excess blood. The desired organs were dissected out, excess moisture and extraneous tissue removed, and the portions weighed out into gelatin capsules, a week's supply at one time. Those not fed immediately were placed in the freezing tray of the



refrigerator where they were frozen and during the 1 week period there were no signs of deterioration.

*Calculation of Results*—The growth response of test animals to varying amounts of riboflavin was determined and from these values a curve of response was constructed (Table II and Fig. 1). Since increments in the amount of riboflavin fed from 2 to 8  $\gamma$  six times a week resulted in relatively greater increments in growth than lower or higher levels, that amount of each tissue was fed which gave growth in this range, preferably at such a level that the growth rate approximated that induced by daily supplements

TABLE II  
*Growth Response to Supplements of Riboflavin Fed Six Times a Week*

Riboflavin	No. of cases	Cumulative gains, probable errors, and coefficients of variation (figures in parentheses)			
		In 1 wk.	In 2 wks.	In 3 wks.	In 4 wks.
$\gamma$		gm.	gm.	gm.	gm.
None	13	1.8 $\pm$ 0.51 (152)	2.1 $\pm$ 0.59 (151)	1.8 $\pm$ 0.66 (198)	1.3 $\pm$ 0.59 (242)
2	15	4.4 $\pm$ 0.35 (45)	6.8 $\pm$ 0.46 (38)	8.3 $\pm$ 0.60 (41)	9.1 $\pm$ 0.59 (37)
4	14	6.1 $\pm$ 0.55 (50)	11.1 $\pm$ 0.61 (31)	15.1 $\pm$ 0.81 (30)	18.7 $\pm$ 0.90 (27)
6	13	9.1 $\pm$ 0.47 (27)	15.2 $\pm$ 0.43 (15)	21.5 $\pm$ 0.69 (17)	24.5 $\pm$ 0.76 (16)
8	11	11.4 $\pm$ 0.60 (26)	19.0 $\pm$ 0.68 (18)	26.0 $\pm$ 0.72 (14)	31.8 $\pm$ 0.78 (12)
12	13	11.7 $\pm$ 1.02 (47)	20.7 $\pm$ 1.14 (30)	27.8 $\pm$ 1.29 (25)	35.2 $\pm$ 1.04 (16)

of 4  $\gamma$  of riboflavin. With the curve of response as a curve of reference and from the gain in weight in 4 weeks of the test animal and of the control paired with it, the results were calculated according to Coward's (8) method. These findings are presented in Table III.

*In Vitro Determination. Extraction*—Different methods have been proposed for the extraction of riboflavin from natural materials. Several of these (4, 10, 21, 25, 31) were here investigated and then replaced by a new one which appears to give quantitative extraction of riboflavin from tissues and to exclude other substances which interfere in the subsequent determination of fluorescence.

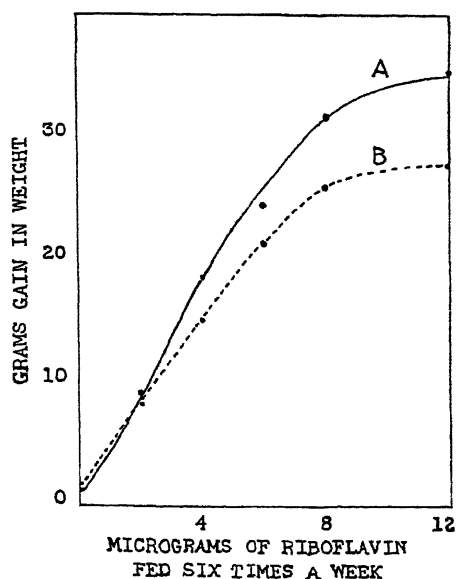


FIG. 1. Growth response to supplements of riboflavin fed six times a week. Curve A represents the total gains in 4 weeks; Curve B, the total gains in 3 weeks.

TABLE III

*Riboflavin Content of Tissues (Micrograms per Gm.) of Rats Reared on Diets 16, 473, 463, and 453*

Tissue		Diet 16	Diet 473	Diet 463	Diet 453
Liver	No. of cases	13	11	22	18
	Mean	32.2	38.9	35.7	43.8
	Probable error	$\pm 2.33$	$\pm 2.99$	$\pm 1.81$	$\pm 2.17$
	Coefficient of variation	39	38	35	31
Skeletal muscle	No. of cases	17	12	11	12
	Mean	3.0	3.8*	3.0	3.5
	Probable error	$\pm 0.14$	$\pm 0.29$	$\pm 0.18$	$\pm 0.26$
	Coefficient of variation	28	39	30	38
Kidney	No. of cases	7	6	6	7
	Mean	26.3	32.3*	32.8	35.0
Heart muscle	No. of cases	3	3	4	2
	Mean	27.0	24.7*	23.0	27.0

\* Calculated on the basis of a 3 week test period because there was not enough tissue for 4 weeks.

Biological assays showed that approximately 43 per cent of the riboflavin in fresh beef liver was extracted by a mixture of 1 volume of methyl alcohol and 4 volumes of 0.125 N hydrochloric acid which was incubated with the liver for 48 hours at 37°; that this amount was increased to 60 per cent by following the above procedure and then refluxing with more of the same reagent for 1 hour; that 60 per cent was extracted by using a mixture of 3 volumes of methyl alcohol and 1 volume of water; and that 73 per cent was extracted if the methyl alcohol-aqueous hydrochloric acid extraction was followed by refluxing with 4 volumes of 60 per cent ethyl alcohol and 1 volume of methyl alcohol. These methods were not considered satisfactory for our purpose. Extracting liver with 60 and 80 per cent acetone produced solutions which exhibited a deep blue fluorescence. Since this color masked the yellow-green fluorescence of riboflavin, the method was rejected.

Riboflavin has been reported (11) to exist in tissues bound to protein in amounts ranging from 70 to 80 per cent of the total. Hence the hydrolysis of the protein in order to free the riboflavin or the riboflavin phosphoric acid presented a possible solution of the problem. Small portions, 0.5 to 2.0 gm., of tissues of rats of corresponding nutritional history to those used in the biological assays (wherever possible rats from the same litters were used) were weighed out, frozen with solid carbon dioxide, ground with sand, and incubated for 20 hours at 37° with 0.3 per cent pepsin in 0.2 per cent hydrochloric acid, a few drops of chloroform being used as preservative. The mixture was then boiled and filtered. The residue was washed with water by decantation. The amount of pepsin solution employed varied from 25 to 50 cc., depending on the weight of tissue taken. The extracts were made up to volume so that the concentration of pepsin and hydrochloric acid would be the same in all cases and so that the concentration of riboflavin would be from 0.2 to 0.6  $\gamma$  per cc. These extracts were clear with a faint yellow color and exhibited the characteristic yellow-green fluorescence of riboflavin when they were illuminated with the 3650 Å. line of the spectrum. Determination of the intensity of fluorescence could be made directly on these extracts. Prior to the final estimation the solutions were shielded from light.

*Apparatus for Determination of Fluorescence*—A Klett fluor-

imeter was used. This instrument is of the two-celled, balanced circuit type so designed that the fluorescence of the unknown solution is measured in terms of the reference standard. The source of incident light is a Type H-4 mercury vapor lamp. Corn-

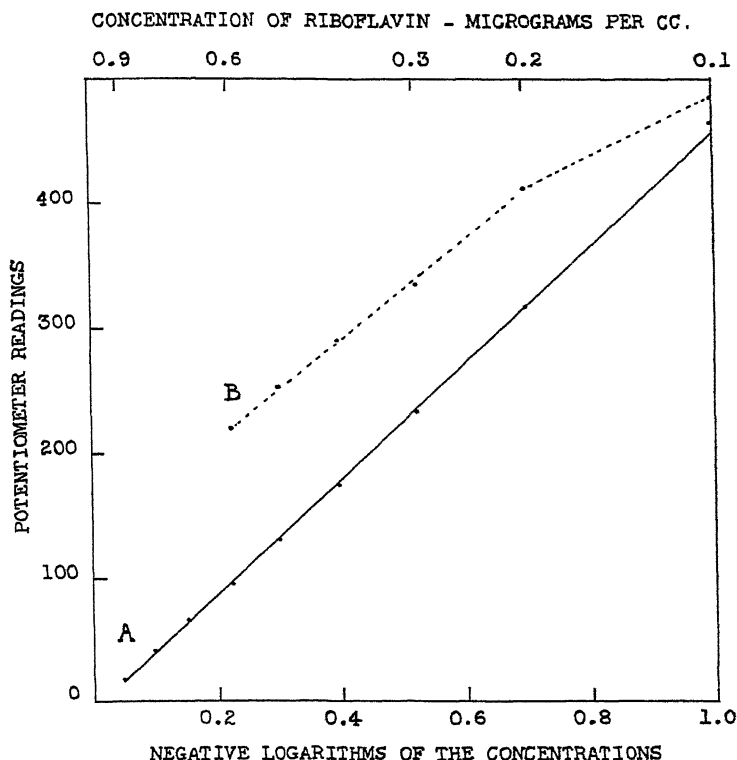


FIG. 2. Relationship between intensity of fluorescence and concentration of riboflavin. Curve A represents the intensity of fluorescence of riboflavin solutions of known concentrations in terms of a solution containing  $1 \gamma$  of riboflavin per cc. as standard; Curve B, riboflavin in pepsin and hydrochloric acid solution read against the standard.

ing glass filters No. 584 were placed between the lamp and the two cells containing the fluorescent solutions and between the latter and the photronic cells were placed Corning glass filters Nos. 440 and 401. This arrangement of filters resulted in the

incident light being mainly of the 3650 Å. line and the light falling on the photronic cells being that from the riboflavin fluorescence, while any scattered incident light was barred. The currents were balanced by means of a potentiometer, with a Leeds and Northrup mirror galvanometer (sensitivity, 0.00036 microampere per mm.)

TABLE IV

*Riboflavin Content of Tissues As Determined by in Vitro Method Here Developed (Micrograms per Gm.) of Rats Reared on Diets 16, 473, 463, and 453*

Tissue	Diet 16		Diet 473		Diet 463		Diet 453	
	No. of cases	Mean and average deviation	No. of cases	Mean and average deviation	No. of cases	Mean and average deviation	No. of cases	Mean and average deviation
Liver.....	8	30.5±2.4	12	39.6±1.6	12	38.9±2.4	11	40.8±2.3
Kidney.....	7	28.7±3.2	5	34.8±2.2	4	31.6±1.0	4	33.6±1.7
Heart muscle....	4	24.6±1.0	4	27.2±1.1	3	28.3±2.5	2	26.6±1.0

TABLE V

*Comparison of Results Obtained by Feeding and in Vitro Methods*

Tissue	Diet No.	Feeding method	Probable error	In vitro	Probable error	Differences between means
		$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Liver	16	32.2	±2.33	30.5	±0.67	-1.7
	473	38.9	±2.99	39.6	±0.35	+0.7
	463	35.7	±1.81	38.9	±0.58	+3.2
	453	43.8	±2.17	40.8	±0.60	-3.0
Kidney	16	26.3		28.7		+2.4
	473	32.3		34.8		+2.5
	463	32.8		31.6		-1.2
	453	35.0		33.6		-1.4
Heart muscle	16	27.0		24.6		-2.4
	473	24.7		27.2		+2.5
	463	23.0		28.3		+5.3
	453	27.0		26.6		-0.4

as a null-point indicator. When the values obtained by measuring the fluorescence of riboflavin solutions of known concentration were plotted in terms of a solution containing 1  $\gamma$  of riboflavin per cc. as standard, a straight line was obtained (the potentiometer scale was logarithmic and so the potentiometer readings were

plotted against the negative logarithms of the concentrations). Also since the readings express a ratio between the intensity of the fluorescence of the standard and of that of the unknown, the potentiometer readings increase as the intensity of the fluorescence of the unknown solution decreases. From Curve A in Fig. 2 it is seen that between 0.9 and 0.1  $\gamma$  per cc. there is a linear relationship between the concentration of riboflavin and the intensity of the fluorescence. Curve B was constructed from readings obtained when riboflavin solutions were treated in the same way as the unknowns (*i.e.*, incubated with 50 cc. of 0.3 per cent pepsin in 0.2 per cent hydrochloric acid for 20 hours at 37°, boiled, filtered, and made up to a volume of 200 cc.) and read against the standard 1  $\gamma$  per cc. of riboflavin solution. In this medium the intensity of fluorescence is less and the linear relationship does not hold at concentrations lower than 0.2  $\gamma$  of riboflavin per cc. Concentrations of the unknown solutions were read from this latter curve. Riboflavin is easily destroyed by light and in order to reduce this source of error the cells containing the standard and the unknown solutions were placed in the light at the same time and the final potentiometer adjustment was made from an approximate one.

### Results

Results obtained by the *in vitro* method are given in Table IV and a comparison of the values obtained by the feeding and *in vitro* methods in Table V.

### DISCUSSION

When the results obtained from the feeding and *in vitro* methods are compared, it is evident that the differences between the two sets of values are statistically insignificant. These differences are of the same order of magnitude as the probable errors of the means obtained by biological assay and are the same or only slightly greater than the average deviations of the means of the chemical determinations. Neither method gives consistently higher or lower results than the other. Probable errors of the means of the *in vitro* determinations, in which the number of cases seemed sufficient for these to be calculated, indicate that the chemical method gives more uniform results than the biological. Further modification of the *in vitro* method is needed before it

can be applied to the determination of riboflavin in skeletal muscle. Extracts of this tissue exhibited a blue fluorescence and the concentrations of riboflavin were too low to be accurately determined by the method which was applied to the extracts of the other tissues.

These figures give evidence of a higher possible riboflavin concentration in liver, kidneys, heart muscle, and skeletal muscle of rats than work reported previously would indicate. However, there are several possible explanations for this difference. Verzár (29) reported that normal rat livers contain from 7.25 to 13.14  $\gamma$  per gm. The livers were finely ground, extracted with water, acidified with hydrochloric acid, boiled, reextracted, and the riboflavin was determined as lumiflavin. Ellinger (9) states that such an extraction process is not complete. Kuhn's experiments (20) show that the transformation of riboflavin into lumiflavin when alkali and light are used is not quantitative or consistent, varying amounts being destroyed depending on the amount taken. This latter fact may account for the low values obtained by Kuhn, Kaltschmitt, and Wagner-Jauregg (19) who report 15 to 17  $\gamma$  per gm. for livers and 0.6  $\gamma$  per gm. for musculature of normally nourished rats. Randoin *et al.* (24) give values for liver, kidneys, and heart of healthy adult rats as being 16.2, 15.5, and 9.39  $\gamma$  of riboflavin per gm. of tissue respectively. Their method was one by the use of which we were unable to obtain quantitative extraction. Determinations by the feeding method have hitherto not been reported in absolute units and, as conversion factors vary under different conditions, no attempt is made to compare those results with ours. The effect of different levels of intake of riboflavin on the amounts found in the tissues is discussed elsewhere.<sup>1</sup>

#### SUMMARY

Existing *in vitro* methods for the determination of riboflavin in tissues were found to be unsatisfactory either because the riboflavin was not quantitatively extracted or because substances interfering in the subsequent estimation of fluorescence were extracted with it. A new method based on the enzymatic hydrolysis

<sup>1</sup> Van Duyne, F. O., and Sherman, H. C., in preparation.

of the tissue protein, extraction with water, and determination of the intensity of the fluorescence of the extract has been developed, tested, and described.

All the present evidence indicates that the method here developed for the extraction of riboflavin and its subsequent measurement *in vitro* is quantitative.

The feeding method used and the *in vitro* method finally developed gave values that were without significant difference.

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# AN ASCORBIC ACID-LIKE REDUCING SUBSTANCE IN THE BUFFY LAYER OF CENTRIFUGED OXALATED BLOOD

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Stephens and Hawley (1), Cuttle (2), and Butler and Cushman (3, 4) have observed a relatively high concentration of an ascorbic acid-like reducing substance<sup>1</sup> in the buffy layer of centrifuged oxalated blood. In normal subjects with plasma ascorbic acid concentrations of 0.2 to 1.5 mg. per 100 cc., Butler and Cushman (4) observed concentrations of apparent ascorbic acid in the buffy layer of 29 to 43 mg. per 100 gm. They showed that this reducing property of the buffy layer of non-leucemic blood is lost with the appearance of scurvy and appears following the administration of ascorbic acid to scorbutic patients and that the reducing capacity of the buffy layer provides an index of physiologically significant vitamin C deficiency (4). These observations and conclusions have been confirmed by Crandon, Lund, and Dill (8) and by Mindlin (9). In patients with leukemia, the concentration of a similar reducing substance in the buffy layer was found to be as high as 140 mg. per 100 gm. when the plasma ascorbic acid was but 0.2 mg. per 100 cc.

The present paper presents further information on the occurrence of this reducing substance in the buffy layer of normal and leucemic blood and on the chemical and physiological evidence concerning its identity.

## EXPERIMENTAL

*Location and Non-Diffusibility of Reducing Substance*—When 10 cc. of normal blood are centrifuged in specially constructed

<sup>1</sup> The reduction was measured against 2,6-dichlorophenol indophenol or methylene blue under conditions used for the estimation of ascorbic acid (5-7, 4).

tubes (4), the buffy layer separates into an upper pure white layer and a pinkish lower layer. As described by Wintrobe (10), the upper layer thus obtained consists almost entirely of platelets, while the lower pinkish layer is predominantly white cells together with some platelets and a few red cells. Analyses of samples from these two layers of normal blood yield approximately the same concentrations of reducing substance in the two layers. Table I shows analyses of (1) the platelet and white cell portions of the buffy layer from two patients with pneumonia and leucocytosis, (2) the upper platelet portion of the buffy layer from

TABLE I

*Apparent Ascorbic Acid Content of Platelet and White Cell Layers of Buffy Layer and Plasma of Centrifuged Oxalated Whole Blood and of White Cells of Spinal, Synovial, and Empyema Fluids*

Source	Diagnosis	Buffy layer		Plasma
		Platelet layer	White cell layer	
		mg. per 100 gm.	mg. per 100 gm.	mg. per 100 cc.
Whole blood.....	Pneumonia	18	16	0.6
" " .....	"	19	17	0.0
" " .....	Postsplenectomy	30		0.9
" " .....	"	22		1.0
" " .....	Purpura		35	1.1
" " .....	"		28	0.9
Spinal fluid.. . . . .			16	
Synovial fluid.....			5	
Empyema " .....			0	

two patients with postsplenectomy platelet counts of approximately 750,000, and (3) the white cell buffy layer from two patients with thrombocytopenic purpura. The data indicate that the reducing substance is present in high concentration in both platelets and white blood cells. Analyses of white cells from spinal fluid, infected joint fluid, and empyema fluid also are shown in Table I. Analyses of white cells from walled-off pus have shown that such disintegrated cells do not contain the reducing substance.

The marked difference in the reducing capacity of the platelets or white cells and of the plasma suggests that this property of these formed elements is dependent upon some constituent of a not

readily diffusible chemical complex. This suggestion is further substantiated by the data of Table II, which give the apparent ascorbic acid content of plasma, of buffy layer, and of whole blood from three leucemic patients and a normal subject. The whole blood analyses were carried out on filtrates obtained by three precipitation procedures, (a) saturation of the blood with CO before precipitation with metaphosphoric acid, thus removing the oxyhemoglobin before hemolysis occurs and preventing oxidation of

TABLE II

*Apparent Ascorbic Acid of Plasma, Buffy Layer, and Whole Blood, According to Method of Precipitation and As Calculated from Its Buffy Layer Content Alone*

Buffy layer per 100 cc. of blood	Plasma	Buffy layer	Whole blood			
	Precipitation with $\text{HPO}_3$	Precipitation with $\text{HPO}_3$	$\text{CO-HPO}_3^*$	Sulfate- tungstate†	$\text{HPO}_3‡$	From buffy layer con- tent§
	(2)	(3)	(4)	(5)	(6)	(7)
cc.	mg. per 100 cc.	mg. per 100 gm.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
6.0	0.3	30	2.6	0.5	0	1.8
3.7	0.2	100	4.0	0.2	0	3.7
6.5	0.3	100	7.0	0.3		6.5
0.6	1.0	30	1.0	0.7	0	0.2

\* CO saturation and  $\text{HPO}_3$  precipitation (4) without oxidation of ascorbic acid.

† Folin's isotonic sulfate-tungstate precipitation (11) without oxidation of ascorbic acid.

‡  $\text{HPO}_3$  precipitation with laking of oxidized red blood cells (4) and oxidation of ascorbic acid.

§ Calculated from the cc. of buffy layer per 100 cc. of whole blood and from the apparent ascorbic acid content of the buffy layer.

ascorbic acid during precipitation (4); (b) suspension of the blood in Folin's isotonic sulfate-tungstate solution (11) followed by sulfuric acid which precipitates the protein without laking the cells or oxidizing ascorbic acid; and (c) direct precipitation of the blood with metaphosphoric acid, which hemolyzes the cells in the presence of oxyhemoglobin and oxidizes ascorbic acid. The data from this last precipitation procedure will be discussed later. From the cc. of buffy layer per 100 cc. of blood and the apparent ascorbic acid content of the buffy layer, the ascorbic acid content

of the whole blood due to its buffy layer content alone has been calculated and recorded in the last column of Table II. From the data, the following is apparent concerning the whole blood analyses. First, that analysis of the CO-HPO<sub>3</sub> filtrate includes the reducing capacity of the buffy layer. Second, the analysis of the isotonic sulfate-tungstate filtrate gives a reducing value that may be assumed to correspond closely with the combined plasma and red cell value (4) and does not include the reducing capacity of the buffy layer. This is particularly striking in the leucemic bloods in which, as shown by the data, 70 to 90 per cent of the apparent whole blood ascorbic acid is in the buffy layer.

The lack of ready diffusibility of a reducing substance from the formed elements of the buffy layer to the surrounding medium is confirmed by the following experiment. A portion of the buffy layer from a sample of centrifuged whole blood was suspended in approximately 100 times its volume of isotonic sulfate-tungstate solution. After 20 minutes the mixture was centrifuged and the supernatant fluid decanted. This fluid, after the addition of sulfuric acid to precipitate the trace of protein, was centrifuged and the supernatant fluid analyzed for apparent ascorbic acid. The washed and centrifuged buffy layer was then ground with metaphosphoric acid and the filtrate analyzed. Another portion of the buffy layer from the centrifuged blood was ground directly with metaphosphoric acid and the filtrate analyzed. Analyses from such experiments are shown in Table III. They demonstrate that there was very little loss of reducing capacity to the supernatant fluid unless the buffy layer had stood for many hours before suspension in the isotonic solution.

*Characterization of Reducing Property of Normal and Leucemic Buffy Layers*—We have previously shown (4) that the time curve of reduction of indophenol and the more specific reduction of methylene blue by metaphosphoric acid filtrates of the buffy layer of normal and leucemic subjects correspond to reduction due to pure ascorbic acid.

As shown by the data of Table II in Column 6 under whole blood, the reducing capacity of both the normal and the leucemic buffy layer is destroyed by laking the oxidized red blood cells during precipitation. This loss, as shown by the data in Column 4, can be prevented by saturating the blood with CO before and

during precipitation. In both respects the behavior is similar to that of ascorbic acid (4, 12-14). Furthermore, analyses of the reducing capacity of metaphosphoric acid filtrates of both normal and leucemic buffy layers by the usual procedure (4) and following treatment with ascorbic acid oxidase prepared according to the method of Fujita and Sakamoto (15) show that the reducing capacity is completely destroyed by the ascorbic acid oxidase.

Though the titration end-point of the Emmerie and van Eekelen method of whole blood analyses (13) is not altogether satisfactory in our hands, analyses of leucemic blood by that method gave, as expected (4), slightly higher values than were obtained by our CO

TABLE III

*Mg. of Apparent Ascorbic Acid per 100 Gm. of Buffy Layer of Centrifuged Human Blood by Analyses of (a) the  $HPO_3$  Filtrate of an Aliquot of the Buffy Layer (4); (b) the  $HPO_3$  Filtrate of an Aliquot of the Buffy Layer Which Had Previously Been Suspended in 100 Parts of Folin's Isotonic Sulfate-Tungstate Reagent for 20 Minutes and Then Centrifuged; and (c) the Tungstic Acid Filtrate of the Sulfate-Tungstate Supernatant Fluid Thus Obtained*

Subject No.	Hrs. after venipuncture	Buffy layer (a)	Washed buffy layer (b)	Supernatant fluid (c)
1	0.5	56	57	2
	48	53	41	13
2	12	20	20	7
	24	23	11	13
3	0.5	14	16	0

saturation method. The reducing substance in the buffy layer of leucemic blood, therefore, is not precipitated with mercuric acetate and is in this respect similar to the reducing substance in the buffy layer of normal blood.

Thus the characterization of the reducing property of the buffy layers of normal and of leucemic bloods by the experiments outlined above has failed to reveal any qualitative difference between the two and has indeed presented striking similarity.

*Physiological Identification*—As mentioned in the introductory paragraph, this reducing property of the buffy layer of human non-leucemic blood is dependent upon the presence of ascorbic acid in

the diet, and its concentration in the buffy layer provides an index of physiologically significant vitamin C deficiency (4, 8, 9). The data of Table IV supplement these findings. They show in experiments on guinea pigs the correlation between the daily intake of ascorbic acid, the concentration of apparent ascorbic acid in the buffy layer of the blood, and the assay of scorbutic changes in the incisor teeth as determined by histological examination by Dr. Otto Bessey (16). Such a correlation between the vitamin C nutritional state of humans and guinea pigs and the reducing property of the buffy layer indicates that the reducing capacity of the non-leucemic buffy layer is due either to ascorbic acid or to a substance metabolically associated with it.

TABLE IV

*Correlation of Ascorbic Acid Ingested per Day by Guinea Pigs, Concentration of Apparent Ascorbic Acid in Plasma and Buffy Layer of Centrifuged Oxalated Blood, and Early Histological Signs of Scurvy in Incisor Teeth*

Ascorbic acid ingested per day	Apparent ascorbic acid		Histological scurvy
	Plasma	Buffy layer	
mg.	mg. per 100 cc.	mg. per 100 gm.	
0.00	0.0	0	++
0.25	0.0	0	++
0.50	0.0	0	++
0.75	0.0	0	+
1.00	0.0	10	?
10.00	0.1	17	Normal

*Osazone of Reducing Substance*—Such physiological identification of the reducing substance of the buffy layer of leucemic blood has not been practical. However, the large volume of this layer in leucemic blood has provided the opportunity of purifying the 2,4-dinitrophenylosazone which may be precipitated from the metaphosphoric acid buffy layer filtrates according to the procedure of Roe and Hall (17). When the dinitrophenylosazone was dissolved in ethyl acetate, precipitated by addition of petroleum ether, washed, dried, and weighed, the actual recovery varied from 45 to 30 per cent of that calculated from the apparent ascorbic acid concentration of the buffy layer. The melting points of these samples of osazone were not altogether satisfactory. Individual

samples obtained from two normal and four leucemic subjects varied from 244–252° (uncorrected), with a range of melting for any one sample of 2° or less. The recovery from pure ascorbic acid solutions by the same procedure was 70 per cent and the melting points of five samples ranged from 249–261° (uncorrected). The melting point obtained by Roe (18) for a product which had not been treated further than by thorough washing with water was 257–259° (corrected).

More satisfactory melting points were obtained when the phenylosazone was recrystallized from alcohol-acetone according to the method of Herbert *et al.* (19). By this method these workers and Drumm, Scarborough, and Stewart (20) and ourselves obtained a melting point of 280–282° (uncorrected) for the crystalline osazone prepared from solutions of pure ascorbic acid. The melting points of the osazone obtained from two lots of leucemic buffy layer were 280–281°, and 278–280°. A mixture of this last preparation and an osazone of pure ascorbic acid had a melting point of 280–282°. While a dinitrophenylosazone could be precipitated from the buffy layer of blood from a normal subject, no such precipitate could be obtained from the buffy layer of blood from a scorbutic patient which contained no apparent ascorbic acid.

#### SUMMARY

The reducing property of metaphosphoric acid extracts of the buffy layer of both normal and leucemic bloods fulfils the commonly used criteria for ascorbic acid as defined by chemical reactions. This property occurs in extracts of both platelets and white blood cells, and apparently is dependent upon some constituent of a not readily diffusible chemical complex. Its concentration in the buffy layer of blood of non-leucemic humans and guinea pigs depends upon the presence of ascorbic acid in the diet and provides an index of physiologically significant ascorbic acid deficiency.

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## THE INFLUENCE OF THIAMINE DEFICIENCY ON CITRIC ACID EXCRETION

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(Received for publication, January 29, 1941)

According to current views on the intermediary metabolism of carbohydrate, both thiamine and citric acid are significant factors in the oxidation of this foodstuff. Thiamine in its phosphorylated form has been shown (Banga, Ochoa, and Peters, 1939) to be required for the oxidative removal of the pyruvic acid produced in the course of carbohydrate breakdown. Likewise, according to Krebs and Johnson (1937), some product (probably pyruvic acid) of carbohydrate metabolism reacts with oxalacetic acid to form citric acid which in turn yields carbon dioxide and a series of simpler dicarboxylic acids before the cycle is repeated. It might be expected, therefore, that the metabolic disturbance characteristic of thiamine deficiency would, in part, make itself felt in an alteration of the "citric acid cycle" and thus possibly in a change in the rate of excretion of citric acid in the urine, inasmuch as it has been shown that even with an adequate allowance of thiamine, dietary carbohydrate exerts a definite influence on the urinary citric acid (Smith and Meyer, 1939).

Studies have been carried out to determine whether or not a relationship between thiamine deficiency and the excretion of citric acid can be demonstrated. A marked increase in citric acid output in the urine of rats has been reported by Krusius and Simola (1938) and also by Krebs (1938). On the other hand Sober, Lipton, and Elvehjem (1940) indicate that severe thiamine deficiency is accompanied by a decrease in urinary citric acid, with a prompt rise of considerable magnitude when the experimental animals (rats) were realimented with thiamine. The data given in the present report have a bearing on this problem.

## EXPERIMENTAL

Eighteen normal rats weighing from 44 to 58 gm. were housed in separate metabolism cages and given a thiamine-deficient diet consisting of sucrose 71, casein<sup>1</sup> 18, salts<sup>2</sup> 3, hydrogenated fat<sup>3</sup> 5, cod liver oil 2, yeast 15. Factors of the vitamin B complex other than thiamine which are needed by rats were provided by a yeast preparation obtained according to Kline, Tolle, and Nelson (1938): 50 gm. of dried yeast<sup>4</sup> were treated with 400 cc. of 0.1 per cent sodium sulfite and brought to pH 4.0 with sulfur dioxide. After standing at room temperature for 5 days, it was lyophilized<sup>5</sup> and finally dried over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator, and ground to a powder.

The funnels of the metabolism cages were washed down daily and the urine collections made in 3 or 4 day periods, at the end of which the rats were weighed and food intake determined.

Citric acid was determined in the urine by the method of Pucher, Sherman, and Vickery (1936), the final measurement being made photoelectrically with a color filter with maximum transmission at 4250 Å.

Six of the rats (Group II) were given in addition to the thiamine-deficient diet 30  $\gamma$  of thiamine daily and their food intake was limited to that of a group of six rats (Group I) without thiamine for which they were paired, fed controls. At the end of seven periods (25 days) Group I was given 5  $\gamma$  of thiamine daily and the experiment on Group II was discontinued. Six other thiamine-deficient rats (Group III) were at this time given 30  $\gamma$  of thiamine daily and paired fed for four periods (14 days) with the six receiving 5  $\gamma$  of thiamine daily.

Twice the daily indicated intake of thiamine, dissolved in 0.2 cc. of water, was placed on top of the food every 2nd day. In Group I after the 5  $\gamma$  of thiamine were given, only slightly more food was put into the food cup than was consumed during the 2 day period

<sup>1</sup> Labco.

<sup>2</sup> Hubbell, Mendel, and Wakeman (1937).

<sup>3</sup> Crisco.

<sup>4</sup> Northwestern Yeast Company.

<sup>5</sup> Through the courtesy of Mr. Harvey Merker, Parke, Davis and Company.

to insure complete consumption of the vitamin. The response in food consumption and in increased body weight shows the effect of the thiamine.

### Results

That the basal diet was deficient in thiamine was shown by the stationary body weights or losses in weight of the rats without thiamine after experimental Period 4, while the addition of 5  $\gamma$  of thiamine *per diem* in Group I and 30  $\gamma$  in Group III was fol-

TABLE I  
*Average Excretion of Citric Acid*

The values are expressed in mg. per kilo of body weight *per diem* based on body weights at the beginning of the indicated period. Each group contained six rats.

Group No.	Period 1, 4 days	Period 2, 4 days	Period 3, 3 days	Period 4, 4 days	Period 5, 4 days	Period 6, 3 days	Period 7, 3 days	Period 8, 3 days	Period 9, 4 days	Period 10, 3 days	Period 11, 4 days
I. Thiamine-free diet*	86	101	49	42	29	20	19	25	29	65	38
II. Thiamine-free diet + 30 $\gamma$ thiamine daily†	74	72	56	67	60	28	17				
III. Thiamine-free diet‡	64	105	64	58	42	37	29	21	62	55	27

\* 5  $\gamma$  of thiamine were fed daily in Periods 8 to 11.

† Paired with Group I as to food intake in Periods 1 to 7.

‡ Paired with Group I as to food intake in Periods 8 to 11 but 30  $\gamma$  of thiamine in addition fed daily.

lowed by prompt resumption of growth at rates of 1 to 3 gm. daily.

In Table I are shown the changes in excretion of citric acid in the various groups of experimental animals. In Groups I and III there is a progressive decrease in the excretion of citric acid by the kidneys as the period of deprivation of thiamine is prolonged. Furthermore, upon realimentation with 5  $\gamma$  of thiamine in Group I and with 30  $\gamma$  in Group III, the output of citric acid increases again. However, that the excretion is a function rather of food intake than of the presence or absence of thiamine is indicated by the parallelism in the amounts of citric acid eliminated by the

rats of Group II when the calories ingested were the same as in Group I but 30  $\gamma$  *per diem* of thiamine were added. Again, when the energy intake is controlled as in the paired feeding of Groups I and III, the amount of thiamine consumed exerted little influence upon the citric acid excretion.

The observations herein discussed do not agree with those of Krusius and Simola (1938), whose experimental ration apparently was deficient in several factors (see Sober, Lipton, and Elvehjem (1940)), nor with those of Krebs (1938), who did not give details of his investigation. Likewise, the present data do not support the contention of Sober, Lipton, and Elvehjem (1940) that thiamine deficiency *per se* is the cause of a decreased output of citric acid. Their experimental procedure differed from that herein described with respect to the details of the diets, though both would appear to be lacking the same essential. Also, there is a difference in the degree of deficiency imposed, only one of the animals in the present study showing polyneuritic spasms. However, while their table is not entirely clear, it does not seem that a real difference exists between the preconvulsive and the convulsive states when such data on individual rats can be compared. A question may also be raised regarding the use of rats fasted 24 hours to provide controls for the effect of food intake on citric acid excretion. It has been our experience that following a change in dietary régime, the excretion of citric acid shows a definite lag over a period of several days, seldom stabilizing during the first 3 days (see Smith and Meyer (1939)). Another question which should be raised is the influence of the acidosis during polyneuritic convulsions; the excretion of citric acid is extremely sensitive to the acid-base balance in the organism (see Smith and Orten (1937)), even citric acid itself when administered orally causing a decreased excretion of citrate (Kuether, Meyer, and Smith, 1940). The result, in this case, would likewise be to depress the output.

#### SUMMARY

A deficiency of thiamine in the diet results in decreased excretion of citric acid in the urine. However, the results of paired, feeding experiments indicate that this decrease is correlated with the diminished intake of food rather than with absence of thiamine *per se*.

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## THE EFFECTS OF DIFFERENT BUFFERS ON THE ACTIVITY OF $\beta$ -AMYLASE

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(Received for publication, January 14, 1941)

In a previous investigation by Ballou and Luck (1), the specific effects of different buffers on the pH optimum of taka-diestase activity and the relative activities of the enzyme at the pH optima and on the acid and alkaline sides of the optima were studied. The present work constitutes an extension of this investigation to  $\beta$ -amylase. The following ten buffers, in systems of constant ionic strength, were used: formate, acetate, propionate, butyrate, valerate, phenyl acetate, phthalate, succinate, phosphate, and citrate. In addition, the activity-pH relationship for a valerate buffer was determined, in which a constant total valerate concentration of 0.07 M was maintained. This was done to permit comparison with the results obtained at constant ionic strength over the same pH range.

Because of the pronounced effects of concentrated urea solutions on proteins, it was thought that it would be of interest to include in this work several runs in the presence of concentrated urea. Gerber (2) reported that, while low concentrations of urea had little effect on diastatic activity, there was marked retardation in higher concentrations. A concentration of 0.133 M urea was found to have a slight inhibitory influence on malt amylase at pH 4.5 by Filipowicz (3). In contrast with these results on amylase, several investigators (4-7) have reported that high concentrations of urea promote the action of the proteolytic enzymes, pepsin, papain, asclepain, and trypsin.

### EXPERIMENTAL

*Preparation of Buffers*—The proportions of buffer acid and sodium hydroxide to be mixed to obtain a desired pH and ionic



strength were determined as described in the report on taka-diastase (1). Equations 3 and 4 of this previous work were employed in the present investigation to calculate the correct proportions of sodium hydroxide and potassium dihydrogen phosphate for the phosphate buffer mixtures; some phosphoric acid was added to obtain pH values lower than 4.5, in which region very little buffering occurred. For the valerate buffer mixtures of constant total valerate concentration (0.07 M), 0.76 cc. of the anhydrous acid was mixed with variable amounts of 1.0 N NaOH and diluted to 100 cc.

*Preparation of Substrate-Buffer Mixtures*—The substrate-buffer mixtures were prepared in an identical manner to that reported in the work on taka-diastase (1), except that a final starch concentration of 1 gm. of dry starch per 100 cc. of solution was used.

The 2 M urea mixtures were prepared in the following manner. To a 100 cc. volumetric flask, calibrated for delivery, were added the desired volume of glacial acetic acid and 5 cc. of 1.0 N NaOH, 50 cc. of starch solution containing 1 gm. of dry starch, 25 cc. of concentrated urea solution containing 12 gm. of urea, and sufficient redistilled water to bring the final volume to 100 cc. In the preparation of the 4 M urea mixtures, 40 cc. of starch solution containing 1 gm. of starch and 50 cc. of urea solution containing 24 gm. of urea were pipetted into the 100 cc. volumetric flask.

*Preparation of  $\beta$ -Amylase*—Several investigators (8-11) consider whole wheat flour an excellent source of  $\beta$ -amylase, and report that hard winter wheat is in most respects superior to barley. The following modified procedure for the isolation of  $\beta$ -amylase includes some steps that were suggested by the papers of Hanes and Cattle (12), van Klinkenberg (10), and Myrbäck and Örtengren (13).

100 gm. of wheat flour, prepared with a hand grist mill, were stirred slowly and thoroughly with 300 cc. of distilled water at room temperature for  $\frac{1}{2}$  hour. The thick suspension was then centrifuged to separate the coarser material. The centrifugate, a cloudy suspension of fine particles, was again centrifuged in cellulose nitrate tubes in an angle centrifuge for about 20 minutes at 4000 R.P.M. at a temperature of 0-5°. The centrifugate (about 200 to 210 cc.) still had a slightly turbid appearance. The remaining steps in the procedure were carried out entirely in the cold room.

An equal volume of cold, redistilled, 95 per cent ethyl alcohol was added to the chilled centrifugate with stirring. Slow stirring was continued for several minutes after the addition of the alcohol. The best results were obtained when both alcohol and centrifugate were chilled thoroughly before mixing. During 15 minutes of standing a flocculent precipitate was brought down by the alcohol. This was centrifuged off with the angle centrifuge and discarded. To the clear centrifugate (about 385 cc.) 1.8 times its volume of cold 95 per cent alcohol was added slowly with stirring. This gave a final alcohol content of approximately 80 per cent by volume. The mixture was stirred slowly for several minutes and allowed to stand for 5 to 10 minutes, during which time a white flocculent precipitate settled out.

The fraction coming down between 50 and 80 per cent alcohol concentration was considered to contain most of the  $\beta$ -amylase (10, 12, 13), and was centrifuged off. The rather well packed sediment in the centrifuge tubes was transferred to a small evaporating dish, broken up into smaller particles, and desiccated in a high vacuum over phosphorus pentoxide, after removal of most of the alcohol by continuous pumping for from 1 to 2 hours.

A creamy white, caked material was obtained after it had remained in the evacuated desiccator overnight. The material was pulverized and stored in a screw top bottle in the cold room. Approximately 0.9 gm. was obtained per 100 gm. of whole wheat flour. The enzyme powder dissolved readily in water and left only traces of insoluble material which settled to the bottom of the solution on standing for a few minutes.

Its activity compared favorably with that reported by the investigators mentioned above (10, 12, 13), and on the whole this process was less complicated and more rapid. Several gm. of the material were prepared in a day. 1 mg. of the enzyme powder was found to produce, at 30° and in the presence of an acetate buffer at pH 5.0, 6.8 mg. of maltose per minute from 100 cc. of 1 per cent starch solution, and 7.25 mg. per minute from a 2 per cent starch solution.

*Preparation of Reaction Mixture*—25 mg. of  $\beta$ -amylase powder were dissolved in several cc. of redistilled water and diluted to 25 cc. For each run 1 cc. of this enzyme solution was pipetted into a 250 cc. Florence flask, and the digestion carried out at 30° in a manner similar to that described in the work with taka-diastrase (1).

A modified Willstätter-Schudel hypiodite method (14, 15) was employed to follow the increase in reducing power of the digest. In several control experiments it was shown that the presence of 2 M urea did not interfere with the stoichiometric oxidation of the carbohydrate reducing groups by the hypiodite reagent.

### Results

Fig. 1 presents the activity-pH curves for  $\beta$ -amylase in the presence of formate, acetate, propionate, butyrate, and valerate buffers, and Fig. 2 the corresponding curves for phenyl acetate, phthalate, succinate, citrate, and phosphate buffers. The reciprocal of the time for the reduction of 2 cc. of 0.05 N  $I_2$  by a 5 cc. aliquot of digest (equivalent to the production of about 26

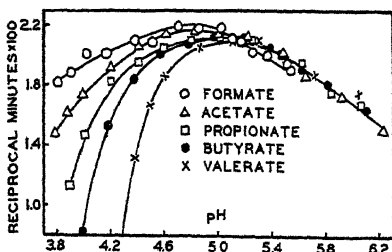


FIG. 1

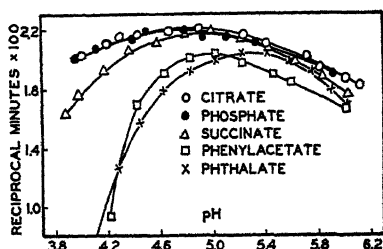


FIG. 2

FIGS. 1 AND 2. Activity-pH curves for  $\beta$ -amylase with the reciprocal of the time for the reduction of 2 cc. of 0.05 N  $I_2$  as the index of activity.

per cent of the theoretical maltose) was accepted as the index of enzyme activity.

Table I presents a list of the buffers and the corresponding pH optima for  $\beta$ -amylase and taka-diastase (1) at a constant ionic strength of 0.05.

In Fig. 1 the pH optimum shifts slightly to the alkaline side, and the relative activity at the optimum diminishes gradually as one ascends the homologous series from formate to valerate. On the alkaline side of the pH optima the activity curves for the monobasic acid buffers coincide, and lie slightly under those for the polybasic acid buffers. On the acid side of the optima, however, the saccharogenic activity of  $\beta$ -amylase varies appreciably with the buffer. Increase of carbon chain length of the buffer acid

exhibits a greater inhibitory influence on  $\beta$ -amylase than on taka-diastase (1) in the acid region.

The curves for phosphate and citrate practically coincide. The relative activity of the enzyme at the optimum in the presence of the two aromatic buffers is less than that for the other buffers. The zones of optimum pH activity for  $\beta$ -amylase in the fatty acid

TABLE I

*Summary of pH Optima for  $\beta$ -Amylase and Taka-Diastase in Presence of Different Buffers*

Buffer	pH optimum for $\beta$ -amylase	pH optimum for taka-diastase
Formate.....	4.7	4.6-5.2
Acetate. . . . .	4.8	5.1
Propionate.....	5.0	5.1
Butyrate . . . . .	5.0	5.1
Valerate. ....	5.2	5.1
Phenyl acetate..	5.0	5.1
Succinate... ..	5.0	5.1-5.3
Phthalate.....	5.3	5.4-5.6
Citrate.....	4.5-4.9	5.1-5.3
Phosphate.....	4.5-4.8	

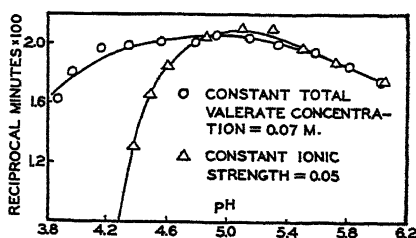


Fig. 3. Activity-pH curves for  $\beta$ -amylase in the presence of a valerate buffer.

buffers are narrower than for taka-diastase, and slightly removed toward the acid side.

Fig. 3 presents two markedly different activity-pH curves for  $\beta$ -amylase with a valerate buffer. The important observation in Fig. 3 is the wide deviation of the curves on the acid side of the optimum. Over the pH range of one curve, a constant ionic

strength of 0.05 was maintained, while the other curve was constructed for a buffer of total, constant valerate concentration (0.07 M). The concentration of 0.07 M valerate was chosen because it was the same as the total valerate concentration at the pH optimum of the activity at constant ionic strength. Wisansky (16) published two similar curves for invertase in an acetate buffer.

That a specific buffer influence on the shape of the activity-pH curve exists is evident from the results obtained in this investigation with  $\beta$ -amylase, and from those of the previous study on taka-diastase (1). It can also be observed that the concentrations of the buffer constituents exert an appreciable effect. From the standpoint of an electrostatic association of the protein ion and the oppositely charged buffer ions, and on the assumption that the pH optimum coincides with the isoelectric point (17), it is not difficult to understand the relatively small influence of the variable buffer anions on the optimum pH. The observed shifts, however, may be accounted for by a reasoning somewhat similar to that advanced by Adair and Adair (18) and Tiselius and Svensson (19); namely, that near the isoelectric point the protein combines to some extent with the buffer ions, preferentially the anions. This argument assumes implicitly that  $\beta$ -amylase and taka-diastase are proteins.

On the alkaline side of the pH optima, where the amphoteric enzyme molecule is increasingly negative in charge, the buffer cation consists only of sodium ion for eight of the buffers, and of a mixture of sodium and potassium ions for the phthalate and phosphate buffers. Hence, a coincidence of the activity curves is to be found in this region. On the acid side of the pH optima, it may be expected that specific influences would be in evidence by virtue of the differences in nature and size of the various oppositely charged buffer anions.

The possibility of a specific action of the neutral buffer acid on the activity of the enzyme must also be considered. A decrease in pH for a given buffer necessarily involves an increasingly neutral acid concentration. It is possible that the relatively higher neutral acid concentration at lower pH values fosters an increasing rate of inactivation of the enzyme (and specifically so) on standing in solution; or it may be that the neutral acid molecules are adsorbed by the protein ions and thus interfere with their enzymic properties.

In Fig. 4 two activity-pH curves for  $\beta$ -amylase and an acetate buffer are presented, the lower one resulting from experiments in the presence of 2 M urea. Owing to the greatly reduced activity in the presence of urea, it was found expedient to use as the index of activity the reciprocal of the time necessary for the reduction of 1.40 cc. of 0.05 N  $I_2$  by a 5 cc. aliquot of digest. Although the

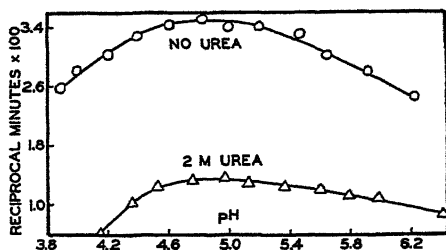


FIG. 4. Activity-pH curves for  $\beta$ -amylase with an acetate buffer in the presence of no urea and 2 M urea.

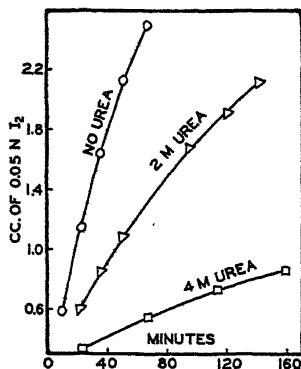


FIG. 5. Time curves for  $\beta$ -amylase and an acetate buffer at the pH optimum in the presence of no urea, 2 M urea, and 4 M urea.

activity was reduced by the urea, the curves remained approximately parallel, with practically no shift in the pH optimum.

Three time curves are plotted in Fig. 5 which illustrate the relative activity of  $\beta$ -amylase at its pH optimum in the presence of no urea, 2 M urea, and 4 M urea. The number of cc. of 0.05 N  $I_2$  reduced by a 5 cc. aliquot of digest is plotted against the time in minutes. These results agree with the findings of Gerber (2) and Filipowicz (3).

## SUMMARY

1. The influence of a number of different buffers on the activity of  $\beta$ -amylase at 30° and an ionic strength of 0.05 was investigated over an approximate pH range of 3.8 to 6.2.

2. The pH optima for  $\beta$ -amylase activity in the presence of formate, acetate, propionate, butyrate, valerate, phenyl acetate, succinate, phthalate, citrate, and phosphate were determined.

3. Variation of the buffer anion was without significant influence on the relative activity of  $\beta$ -amylase at the pH optima, except for a slight inhibitory effect of phenyl acetate and phthalate.

4. The activity-pH curves approximately coincided on the alkaline side of the pH optima, whereas on the acid side a marked specific buffer influence was manifested.

5. A rapid and convenient method is described for the preparation from hard winter wheat of  $\beta$ -amylase in the form of an active, water-soluble powder.

6. High concentrations of urea were found to inhibit the saccharogenic action of  $\beta$ -amylase on starch.

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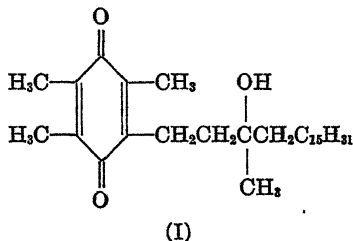
# VITAMIN E ACTIVITIES OF SOME COMPOUNDS RELATED TO $\alpha$ -TOCOPHEROL

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During the course of an extensive investigation of the anti-hemorrhagic activity of vitamin K-like substances, certain synthetic methods were developed which made it possible to prepare some interesting compounds closely related to  $\alpha$ -tocopherol (1-3). These compounds have been tested for vitamin E activity, and a discussion of the results is now presented.



At the outset of the investigation the preparation of pure  $\alpha$ -tocopherylquinone (I) was undertaken, as the biological activity of this compound has been a matter of some discussion (4). More recent results from various laboratories (5), however, indicate that  $\alpha$ -tocopherylquinone shows no vitamin E activity at doses up to 30 mg. as compared to 3 mg., the active dose of  $\alpha$ -tocopherol. Inasmuch as the method of isolating and purifying the quinone developed in the Merck laboratory (6) differs from those previously described, the present assays have a significant bearing on the problem.

Our method of isolating  $\alpha$ -tocopherylquinone utilizes the fact that its hydroquinone is sparingly soluble in petroleum ether, and, as in the purification of vitamin K<sub>1</sub> (7), the hydroquinone



may be obtained in a high state of purity by repeated washings with this solvent. The pure dihydro form can then be converted to the quinone by mild oxidation. Samples of tocopherylquinone purified in this manner showed no vitamin E activity in doses as high as 100 mg., as shown in Table I.

The inactivity of  $\alpha$ -tocopherylquinone at high dose levels is striking, considering the ease with which this quinone can be quantitatively reconverted to  $\alpha$ -tocopherol by heating with acidic reducing agents for a short time.

TABLE I  
*Summary of Vitamin E Assays\**

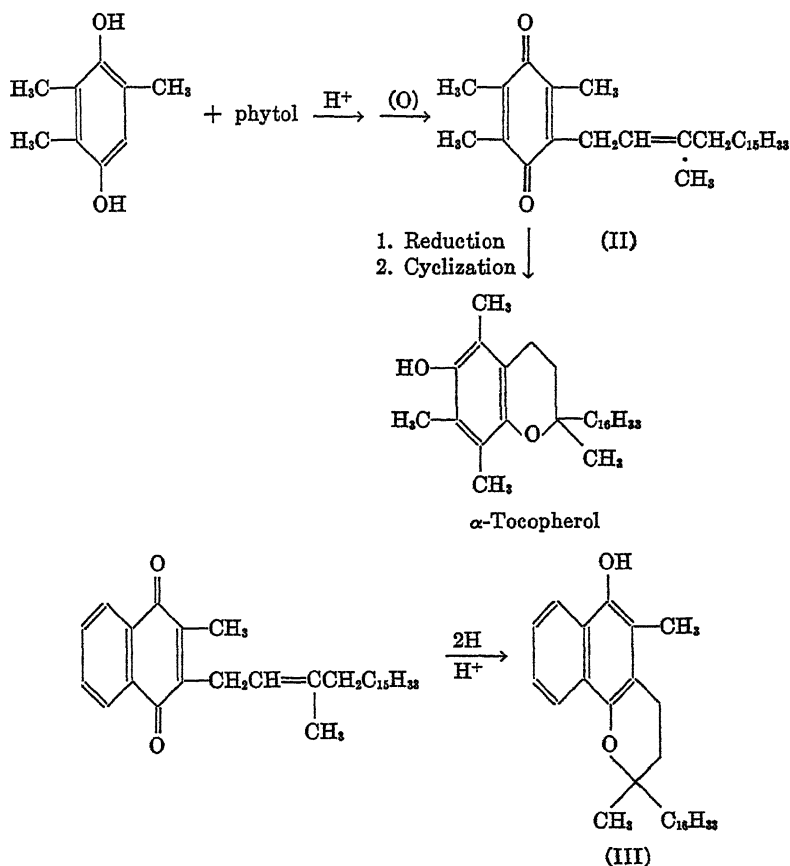
Compound	Dose level	No. of animals	No. of resorptions	No. of litters
	mg.			
$\alpha$ -Tocopherylquinone (I)	15	8	8	0
	100	8	8	0
2,3,5-Trimethyl-6-phytyl-1,4-benzoquinone (II)	25	8	8	0
	100	6	5	1
$\alpha$ -Tocopherol prepared from 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone	3	7	1	6 (1 poor)
$\alpha$ -Tocopherol prepared from $\alpha$ -tocopherylquinone	3	9	3	6
Naphthotocopherol (III)	25	7	0	7 (1 poor)
	10	7	5	2 (Poor)
2,3,5-Trimethyl-6-(dihydrophytyl)-1,4-benzoquinone (IV)	25	7	7	0

\* The assays were carried out at the University of California.

Owing to the pronounced tendency of  $\alpha$ -tocopherol to undergo an irreversible oxidation to  $\alpha$ -tocopherylquinone, it seems possible that a large portion of this vitamin is changed during metabolism to the inactive tocopherylquinone or one of its further transformation products. This deactivation may account for the fact that the curative dose of  $\alpha$ -tocopherol is so much larger than those found for the other known vitamins.

Inasmuch as no two other vitamins are so similar from a structural and synthetic point of view as are vitamin K<sub>1</sub> and  $\alpha$ -tocopherol, it seemed of interest to assay the benzo analogue of vitamin K<sub>1</sub>; namely, 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone (II) (3). The latter, prepared by the condensation of 2,3,5-trimethyl-

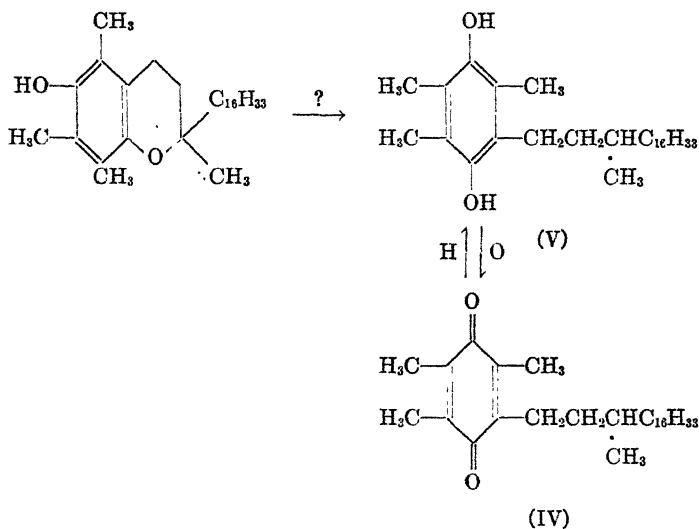
1,4-benzohydroquinone and phytol under mild conditions, is readily converted by acidic reducing agents to  $\alpha$ -tocopherol. This conversion, the basis of a new synthesis of  $\alpha$ -tocopherol, was established by chemical methods (3) and by biological assays. The trimethylphytylbenzoquinone, however, showed no activity at a dose of 100 mg. It is apparent that, as in the case of  $\alpha$ -tocopherylquinone (I), the animal body cannot transform the substance to  $\alpha$ -tocopherol by reduction and cyclization.



Like 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone (II), vitamin K<sub>1</sub> may be converted by acidic reducing agents to naphthotocopherol (III) (8) which has moderate vitamin E activity. This

substance, the first chroman containing the naphthalene nucleus to be assayed, is completely active at a dose of 25 mg., but only slightly so at 10 mg. Naphthotocopherol, as has been previously reported, also shows a slight vitamin K activity (8), its effective dose in the chick assay being about 500 times that of vitamin K<sub>1</sub>.

The fourth compound of this series to be examined was 2,3,5-trimethyl-6-( $\beta,\gamma$ -dihydrophytyl)-1,4-benzoquinone (IV) (3). This compound was selected in view of the possibility that during the metabolism of  $\alpha$ -tocopherol a reductive cleavage might occur to yield the biologically active hydroquinone (V) which may then function as part of an oxidation-reduction system. There appears to be no foundation for this possibility, as the benzoquinone (IV) was found to be ineffective at a dose of 25 mg. (Table I).



The chemical similarity of vitamin K<sub>1</sub> and  $\alpha$ -tocopherol suggests that the two substances may have a somewhat similar origin in nature. It is conceivable that in the biogenesis of both phytol condenses with the simple benzohydroquinone or naphthohydroquinone, as in the laboratory, to give the corresponding phytylhydroquinones. The naphthohydroquinone derivative because of its low oxidation-reduction potential (9) and ease of dehydrogen-

ation in air to vitamin K<sub>1</sub> may then escape cyclization to naphthotocopherol. The benzohydroquinone analogue, however, being much more stable in air (higher oxidation-reduction potential and slower rate of air oxidation) would be more disposed to undergo cyclization to  $\alpha$ -tocopherol. It is interesting to note that  $\alpha$ -tocopherol and vitamin K<sub>1</sub> frequently occur together in plants. Possibly naphthotocopherol will be found present to some extent as a component of vitamin E concentrate. It should be pointed out that the activity of naphthotocopherol is about one-third that of  $\beta$ - and  $\gamma$ -tocopherol.

#### SUMMARY

1. Neither pure  $\alpha$ -tocopherylquinone nor 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone exhibits any vitamin E activity at dose levels of 100 mg., notwithstanding the fact that both substances may be readily converted to  $\alpha$ -tocopherol in the laboratory by acidic reducing agents.

2. 2,3,5-Trimethyl-6-( $\beta$ , $\gamma$ -dihydrophytyl)-1,4-benzoquinone has no vitamin E activity at a dose of 25 mg.

3. Naphthotocopherol, prepared from vitamin K<sub>1</sub>, has moderate vitamin E activity.

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## AZLACTONES

### IV. SYNTHESIS OF $\alpha$ -AMINO- $\beta$ -THIOL-*n*-BUTYRIC ACIDS

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In previous papers (1, 2) Carter *et al.* described the preparation of benzoyl- $\alpha$ -aminocrotonic acid azlactone I. This compound reacts with sodium methylate to give an addition product consisting almost entirely of benzoyl-*dl*-O-methylthreonine (1). Our object in the present work was to study the reaction of the azlactone with benzyl mercaptan, and to attempt the conversion of the addition product into  $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acid. It has been suggested (3) that this amino acid may be an intermediate in the conversion of methionine into cystine.

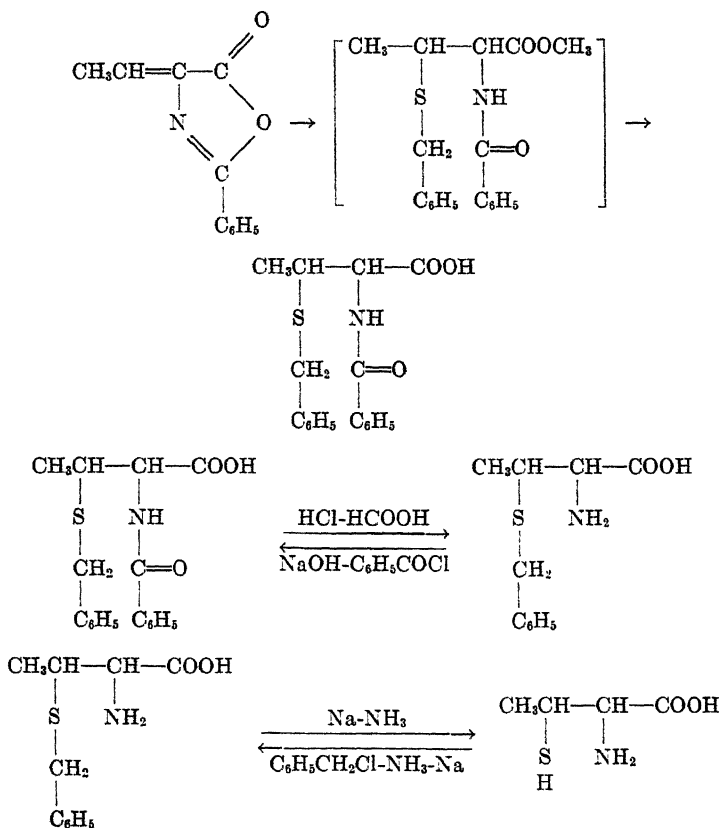
Nicolet (4) and others (*cf.* Mayo and Walling (5)) have studied the addition of mercaptans to  $\alpha, \beta$ -unsaturated ketones and esters. Nicolet (6) has also described the addition of *p*-tolyl mercaptan to benzoyl- $\alpha$ -aminocinnamic acid azlactone. In the last reaction, no mention was made of yields nor of the possibility of obtaining a mixture of the two racemic forms of the addition product.

From the reaction of benzyl mercaptan and benzoyl- $\alpha$ -aminocrotonic acid azlactone I we obtained a mixture of benzoyl-*dl*- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acids A and B<sup>1</sup> in 80 per cent yield. The composition of the mixture varied with the reaction temperature. At  $-30^\circ$  approximately 70 per cent of Benzoyl

<sup>1</sup> Two racemic  $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acids are possible, corresponding to *dl*-threonine and *dl*-allothreonine. Since we have not determined the configuration of these thiolamino acids, it is not possible to assign them names expressing their structural relationship to threonine. In this paper the benzoyl-*dl*- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid melting at  $145-147^\circ$  and the compounds derived from it will be designated by A; the isomeric compounds by B. For the sake of brevity the benzoyl- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acids will be called Benzoyl Derivatives A and B, and the  $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acids will be called Amino Acids A and B.

Derivative A and 30 per cent of Benzoyl Derivative B were obtained. At  $+10^\circ$  the product consisted of approximately 55 per cent of Benzoyl Derivative A and 45 per cent of Benzoyl Derivative B. The benzoyl derivatives were separated in excellent yield by conversion to the  $\beta$ -phenethylamine salts. As in the case of similar compounds (7), the more soluble, lower melting benzoyl derivative gave the less soluble, higher melting  $\beta$ -phenethylamine salt.

We investigated also the addition of benzyl mercaptan to methyl benzoyl- $\alpha$ -aminocrotonate. The yield and composition of the resulting product were the same as those obtained from the azlactone under similar conditions.



Benzoyl Derivatives A and B were converted to Amino Acids A and B, respectively, by removing first the benzoyl group, then the benzyl. Due to their insolubility the benzoyl derivatives were not readily hydrolyzed by refluxing in aqueous hydrochloric acid. However, in a mixture of hydrochloric and formic acids the hydrolysis proceeded smoothly. The benzyl groups were removed by reduction with sodium in liquid ammonia (8). The amino acids obtained were characterized by reconversion to the original benzoyl- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acids.

The reactions involved are shown in the accompanying equations.

Each of the  $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acids gives the characteristic sulfhydryl tests (nitroprusside, ferric chloride, copper sulfate), reduces the theoretical amount of iodine when titrated in 90 per cent acetic acid (9), and yields theoretical nitrogen values in the Van Slyke determination when potassium iodide is added to the reagent (10). Each of the amino acids gives the characteristic color when Lugg's (11) modification of the Sullivan test for cystine is used. When compared in a Cenco-Sanford-Sheard photoelectric colorimeter, with the green filter, Amino Acid B gave approximately the same intensity of color as an equivalent amount of cysteine, while Amino Acid A gave only 20 per cent as much.

#### EXPERIMENTAL

*Addition of Benzyl Mercaptan to Azlactone I*—5 gm. of sodium were dissolved in 1 liter of anhydrous methyl alcohol and 118 cc. of benzyl mercaptan were added. The reaction mixture was stirred and kept at 5–10°, while a solution of 187 gm. of benzoyl- $\alpha$ -aminocrotonic acid azlactone I in 1 liter of benzene was added slowly. After the addition was completed, the cooling bath was removed and the solution was stirred for 1 hour. The solution was acidified to Congo red with 0.5 N hydrochloric acid and was concentrated to a syrup *in vacuo*. The syrup was dissolved in a hot mixture of 2500 cc. of glacial acetic acid, 750 cc. of concentrated hydrochloric acid, and 1500 cc. of water. The solution was refluxed for 50 minutes and allowed to cool overnight. The crystalline precipitate was removed by filtration and washed with 50 per cent acetic acid and then with water, yielding 230 gm. (70



per cent) of a mixture of Benzoyl Derivatives A and B. The neutral equivalent of this material was 330 (calculated, 329).

An additional 35 gm. (10 per cent) of benzoyl derivative were obtained by concentrating the mother liquor to dryness *in vacuo*, benzoylating the residue with 35 cc. of benzoyl chloride and 350 cc. of 2 N sodium hydroxide, and isolating the benzoyl derivatives in the usual manner.

*Addition of Benzyl Mercaptan to Methyl Benzoyl- $\alpha$ -Aminocrotonate*—When pure methyl benzoyl- $\alpha$ -aminocrotonate I was treated with benzyl mercaptan exactly as described for the azlactone, the product obtained was identical in yield and composition.

The methyl ester used in this experiment was prepared by dissolving 14.2 gm. of benzoyl- $\alpha$ -aminocrotonic acid I in 75 cc. of absolute methyl alcohol containing 5 cc. of concentrated sulfuric acid. The mixture was refluxed for 2.5 hours, allowed to stand overnight, and then poured onto ice. The precipitate was removed by filtration, dissolved in ether, and extracted quickly with cold dilute sodium hydroxide. The ether solution was dried and the ether was evaporated. The residue was recrystallized from high boiling petroleum ether, giving 10.6 gm. (70 per cent) of methyl benzoyl- $\alpha$ -aminocrotonate I, melting at 78–80°.

$C_{12}H_{13}O_3N$ . Calculated, N 6.39; found, N 6.16

Hydrolysis of the ester yielded pure benzoyl- $\alpha$ -aminocrotonic acid I.

*Separation of Benzoyl Derivatives A and B*—100 gm. of the mixture of Benzoyl Derivatives A and B were extracted with 800 cc. and 200 cc. portions of boiling benzene. The residue consisted of 26 to 30 gm.<sup>2</sup> of practically pure Benzoyl Derivative B, melting at 178–185° and giving a neutral equivalent of 330. This material was recrystallized from a mixture of 400 cc. of benzene and 35 cc. of absolute alcohol. After cooling for several days, 22 gm. of material melting at 181–186° were obtained.

$C_{12}H_{11}O_3NS$ . Calculated, N 4.26; found, N 4.38

The  $\beta$ -phenethylamine salt of this compound was prepared as described below. After two recrystallizations, the melting point

<sup>2</sup> When the addition of benzyl mercaptan was carried out at –30°, only 14 gm. of Benzoyl Derivative B were obtained at this point, although the total yield was the same.

of the salt was unchanged (147–150°), and the recovered Benzoyl Derivative B melted at 181–187°. These data leave no doubt as to the purity of the compound.

The combined benzene extracts described above were cooled, and 65 gm. of a mixture of Benzoyl Derivatives A and B melting at 138–170° were obtained. This material was dissolved in 800 cc. of hot ethyl acetate, and 25 cc. of  $\beta$ -phenethylamine were added with stirring. After 3 hours at room temperature, the mixture was filtered. The product (76 gm.) was dissolved in a hot mixture of 500 cc. of ethyl acetate and 200 cc. of absolute alcohol. The solution was cooled several hours, giving 66 gm. of the  $\beta$ -phenethylamine salt of Benzoyl Derivative A, melting at 166–168°. The melting point of the salt was not raised by further recrystallization.

The salt was suspended in 1 liter of ether in a separatory funnel and 200 cc. of 10 per cent hydrochloric acid were added. The suspension was shaken vigorously until the salt was decomposed. The ether layer was separated, dried, and evaporated to dryness. The residue was recrystallized from 800 cc. of benzene, giving 42 gm. of Benzoyl Derivative A melting at 145–147°.

$C_{18}H_{19}O_2NS$ . Calculated.	N 4.26,	neutral equivalent	329
Found.	" 4.20,	" "	329

The two filtrates from the amine salts were combined and evaporated. The residue was decomposed as above, giving a mixture of Benzoyl Derivatives A and B. This was extracted with 200 cc. of boiling benzene, leaving a residue of 8 gm. of pure Benzoyl Derivative B. The benzene filtrate on cooling deposited 10 gm. of a mixture of benzoyl derivatives which was added to the next run.

*Hydrolysis of Benzoyl Derivatives A and B*—16.5 gm. of Benzoyl Derivative B were suspended in a mixture of 250 cc. of commercial 85 per cent formic acid, 250 cc. of concentrated hydrochloric acid, and 250 cc. of water. The mixture was refluxed vigorously for 4 hours, at which time some solid was still undissolved. The mixture was evaporated *in vacuo* to dryness, and extracted with two 200 cc. portions of hot high boiling petroleum ether. The residue was extracted with 200 cc. of hot water and filtered from 5.9 gm. of unchanged Benzoyl Derivative B. The aqueous solution was neutralized with ammonium hydroxide, concentrated to

approximately 50 cc., and cooled overnight. The precipitate was removed by filtration and washed, yielding 5.1 to 5.5 gm. (70 to 75 per cent) of  $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid B melting with decomposition at 202–204°.

$C_{11}H_{15}O_2NS$ . Calculated, N 6.22; found, N 6.09

Benzoyl Derivative A was hydrolyzed by the same procedure, except that the reaction mixture was refluxed only 3 hours. On extraction with hot water 4.8 gm. of unchanged Benzoyl Derivative A remained. 6.0 gm. (75 per cent) of  $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid A, melting with decomposition at 197–199°, were obtained on neutralization and concentration of the filtrate.

$C_{11}H_{15}O_2NS$ . Calculated, N 6.22; found, N 6.17

*Reduction of the  $\alpha$ -Amino- $\beta$ -Benzylthio-*n*-Butyric Acids*—15 gm. of  $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid B were dissolved in 250 cc. of liquid ammonia and treated with small pieces of sodium in the usual manner. Slightly more than 2 moles of sodium per mole of amino acid were required to produce a permanent blue color. Ammonium chloride was added in small portions until the blue color disappeared and then an additional 7 gm. were added. The ammonia was allowed to evaporate, the last traces being removed *in vacuo*. 250 cc. of ether and 5 cc. of concentrated hydrochloric acid were added. The solid material was broken up thoroughly and the flask was warmed on the steam cone for a few minutes. The ether was decanted and the residue was extracted again with ether. Subsequent operations were carried out in an atmosphere of nitrogen. The residue was extracted with three 100 cc. portions of warm absolute alcohol containing a few drops of concentrated hydrochloric acid. The combined alcohol extracts were concentrated to dryness *in vacuo*. The residue was dissolved in 80 cc. of absolute alcohol and 800 cc. of anhydrous ether were added. The flask was cooled overnight and the precipitate was removed, washed with ether, and dried, giving 9.8 gm. of almost pure hydrochloride of Amino Acid B. This was dissolved in 300 cc. of alcohol and 3.8 cc. of concentrated ammonium hydroxide were added. On cooling, 6.4 gm. (71 per cent) of pure Amino Acid B, melting with decomposition at 203–204°, were obtained.

$C_4H_9O_2NS$ . Calculated. N 10.36

Found. " 10.51 (Dumas); N 10.47 (Van Slyke)

Titration with iodine in 90 per cent acetic acid gave a sulphydryl content of 97 per cent.

Amino Acid A was obtained by the same procedure. 15 gm. of  $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid A were dissolved in 400 cc. of liquid ammonia. When sodium was added, a purple color appeared and then a copious white precipitate separated, necessitating stirring by hand. The precipitate disappeared on addition of ammonium chloride. 10.6 gm. of the hydrochloride of Amino Acid A were obtained. This gave 6.7 gm. (75 per cent over-all yield) of Amino Acid A, melting with decomposition at 203–205°.

$C_9H_{11}O_2NS$ . Calculated. N 10.36

Found. " 10.35 (Dumas); N 10.30 (Van Slyke)

Iodine titration in 90 per cent acetic acid gave a sulphydryl content of 98 per cent.

*Conversion of Amino Acids A and B to Benzoyl Derivatives A and B, Respectively*—1.35 gm. of pure Amino Acid A were dissolved in 100 cc. of liquid ammonia, and metallic sodium was added until the characteristic blue color appeared. The blue was just removed with ammonium chloride, and 1.5 cc. of benzyl chloride were added. The ammonia was evaporated, and the residue extracted with ether and dissolved in 50 cc. of water. The solution was acidified to Congo red, filtered, and neutralized to litmus with ammonium hydroxide. When concentrated and cooled, 1.7 gm. of  $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acid A were obtained. 1.12 gm. of this material were dissolved in 7.5 cc. of 1 N sodium hydroxide and treated with 2.1 gm. of benzoyl chloride and 35 cc. of 1 N sodium hydroxide in the usual manner. The resulting solution was acidified to Congo red. The precipitate was removed by filtration, washed, dried, and extracted with hot high boiling petroleum ether. The product consisted of 1.4 gm. (64 per cent over-all yield) of Benzoyl Derivative A melting at 143–145°.

By exactly the same procedure Amino Acid B was converted to Benzoyl Derivative B, melting at 177–182°, in 62 per cent over-all yield.

#### SUMMARY

1. A mixture of the benzoyl- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acids is obtained in 80 per cent yield by the addition of benzyl mercaptan to benzoyl- $\alpha$ -aminocrotonic acid azlactone I. The

two benzoyl derivatives are readily separated through the  $\beta$ -phenethylamine salts.

2. The  $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acids are prepared in 50 per cent yield from the corresponding benzoyl- $\alpha$ -amino- $\beta$ -benzylthio-*n*-butyric acids.

3. Some properties of the thiolamino acids are described.

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## AZLACTONES

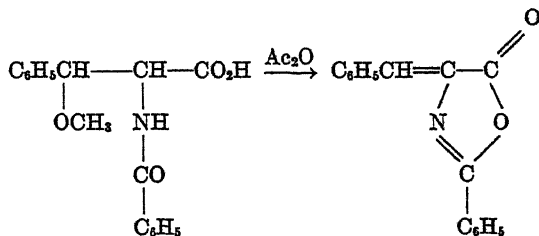
### V. PREPARATION OF BENZOYL- $\alpha$ -AMINOCINNAMIC ACID AZLACTONES I AND II. THE USE OF $\beta$ -PHENETHYLAMINE IN THE PURIFICATION OF $\alpha$ -AMINO- $\beta$ -METHOXY (HYDROXY) ACIDS

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(Received for publication, January 16, 1941)

Carter and Stevens (1) obtained two benzoyl- $\alpha$ -aminocrotonic acid azlactones on treating benzoyl-*dl*-O-methylallothreonine with acetic anhydride. This was the first reported isolation of the geometric isomers of an  $\alpha,\beta$ -unsaturated azlactone. In a continuation of this work we have obtained the corresponding benzoyl- $\alpha$ -aminocinnamic acid azlactones by the action of acetic anhydride on benzoyl-*dl*-O-methylphenylserine B.<sup>1</sup>



Benzoyl- $\alpha$ -aminocinnamic acid azlactone I,<sup>2</sup> melting at 165–166°, has been studied extensively by Erlenmeyer (3, 4). Azlactone II<sup>2</sup> melting at 146–148° has not been described previously.

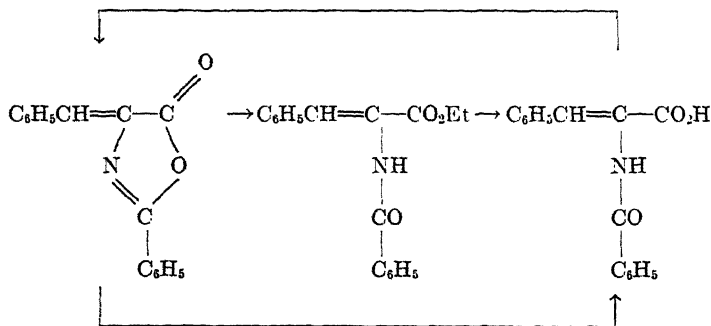
<sup>1</sup> Two benzoyl-*dl*-O-methylphenylserines, melting at 153–154° and 220–222° respectively, were described by Carter and Van Loon (2). The lower melting benzoyl derivative and the corresponding amino acid will be designated by A; the isomeric compounds by B.

<sup>2</sup> For convenience the azlactone melting at 165–166° and the benzoyl- $\alpha$ -aminocinnamic acid and ester derived from it will be designated as I; the isomeric azlactone, acid, and ester will be designated as II.

Azlactone I is stable, while Azlactone II is rapidly converted into Azlactone I by the action of pyridine at room temperature. In this respect the cinnamic acid azlactones resemble the crotonic acid azlactones. However, benzoyl- $\alpha$ -aminocinnamic acid azlactone II is much more stable toward heat than is the corresponding benzoyl- $\alpha$ -aminocrotonic acid azlactone. It should be noted that a shift in the position of the double bonds in benzoyl- $\alpha$ -aminocinnamic acid azlactone is not possible. Therefore the two forms must be geometric and not structural isomers.

In view of the similar results obtained in the two cases studied, it seems probable that  $\alpha$ -amino- $\beta$ -methoxy (hydroxy) acids will generally yield two isomeric  $\alpha,\beta$ -unsaturated azlactones when treated with acetic anhydride.

Considerable difficulty was encountered in purifying benzoyl- $\alpha$ -aminocinnamic acid azlactone II, since it is more soluble than Azlactone I and is present in smaller amounts in the reaction product. Attempts to purify Azlactone II by fractional crystallization were unsuccessful, yielding products melting between 125–140°. However, relatively pure Azlactone II was obtained by another method. Preliminary studies had shown that benzoyl- $\alpha$ -aminocinnamic acid I and both of the benzoyl- $\alpha$ -aminocrotonic acids are converted into the corresponding azlactones with little or no isomerization. Therefore pure benzoyl- $\alpha$ -aminocinnamic acid azlactone I and crude Azlactone II were carried through the reactions shown in the equations.



Azlactone I yielded a single ethyl benzoyl- $\alpha$ -aminocinnamate (Ester I, melting at 147–149°) and a single benzoyl- $\alpha$ -aminocin-

namic acid (Acid I, melting at 229–230°). Erlenmeyer (3) has prepared these compounds by less satisfactory methods. Crude Azlactone II gave a mixture of ethyl benzoyl- $\alpha$ -aminocinnamates I and II, from which a considerable amount of Ester I was separated. The residue, on hydrolysis, yielded a mixture of benzoyl- $\alpha$ -aminocinnamic acids from which Acid II, melting at 199–200°, was obtained in a pure form. On treatment with acetic anhydride, Acid II yielded Azlactone II, melting at 146–148°. A mixture of Azlactones I and II melted at 125–135°. It would appear, therefore, that this method yields relatively pure benzoyl- $\alpha$ -aminocinnamic acid azlactone II.

In a further extension of the work we have studied the reaction of benzoyl-*dl*-O-methylphenylserine A and of benzoyl-*dl*-O-methylthreonine with acetic anhydride. These compounds yield approximately the same mixture of azlactones as do their respective diastereoisomers.

In the preparation of the benzoyl-*dl*-O-methylphenylserine A used in this work, it was discovered that the material melting at 153–154° is not entirely free of Benzoyl Derivative B, and that it cannot be purified further by recrystallization. In an effort to obtain pure benzoyl-*dl*-O-methylphenylserine A the properties of certain derivatives were investigated. It was discovered that the  $\beta$ -phenethylamine salts of benzoyl-*dl*-O-methylphenylserines A and B are beautifully crystalline solids which are readily recrystallized. Furthermore, there is a striking reversal in the relative solubility of the isomers in going from the benzoyl derivatives to the salts. The more soluble, lower melting benzoyl derivative gives the less soluble, higher melting  $\beta$ -phenethylamine salt, thus furnishing a simple method of purifying Benzoyl Derivative A. The pure compound thus obtained melts sharply at 166–167°.

The same reversal in relative solubility and melting points of the free acids and their  $\beta$ -phenethylamine salts was observed in the following pairs of isomers: N-benzoylthreonine and N-benzoylallothreonine; benzoyl-O-methylthreonine and benzoyl-O-methylallothreonine; carbobenzoxy-*dl*-O-methylphenylserines A and B. We have recently used this effect to advantage in preparing the two *dl*- $\alpha$ -amino- $\beta$ -thiol-*n*-butyric acids. It seems possible that  $\beta$ -phenethylamine may be generally useful in separating diastereoisomers of this type.



## EXPERIMENTAL

*Azlactonization of Benzoyl-dl-O-Methylphenylserines A and B*—15 gm. (0.05 mole) of benzoyl-dl-O-methylphenylserine B were suspended in 75 cc. of acetic anhydride and the mixture was heated on the steam cone until the benzoyl derivative had dissolved (10 to 15 minutes). The solution was cooled in an ice bath, giving 6 to 7 gm. of benzoyl- $\alpha$ -aminocinnamic acid azlactone I. The filtrate was poured into ice water with vigorous stirring. A light yellow solid slowly separated as the acetic anhydride hydrolyzed. This material was removed by filtration, washed with water, and air-dried, yielding 4.6 gm. of a somewhat sticky solid melting at 124–130°. Recrystallization of this product from benzene, benzene-alcohol, or benzene-petroleum ether gave impure benzoyl- $\alpha$ -aminocinnamic acid azlactone II melting between 124–140°.

Several variations in the procedure were tried without appreciably increasing the amount of Azlactone II obtained. The yield of Azlactone II is decreased if the heating is prolonged or if large quantities of benzoyl derivative are worked up at one time.

Benzoyl-dl-O-methylphenylserines A and B are also azlactonized by the action of benzoyl chloride or of acetic anhydride in pyridine under the conditions previously described (5). Only the stable Azlactone I is obtained in these reactions.

*Ethyl Benzoyl- $\alpha$ -Aminocinnamates I and II*—The benzoyl- $\alpha$ -aminocinnamic acid azlactones were alcoholized by the method of Nicolet (6) with certain modifications, including a reduction of the reaction time from 1 hour to 3 minutes. 20 gm. of Azlactone I were suspended in 80 cc. of benzene and 10 cc. of 1 N sodium ethylate were added. The azlactone rapidly dissolved and ethyl benzoyl- $\alpha$ -aminocinnamate I began to separate almost immediately. After 3 minutes an excess of dilute hydrochloric acid was added and the mixture was vigorously shaken. The white solid was removed by filtration, washed with water, and dried, giving 20 gm. (84 per cent) of almost pure Ester I melting at 142–146°. An additional 1.5 gm. of crude Ester I were recovered on concentration of the benzene layer.

15 gm. of crude Azlactone II, melting at 124–135°, were treated as described above. No solid separated during the reaction. On acidification and cooling for several hours in an ice bath a solid crystallized. This was removed by filtration, yielding 5 gm.

of ethyl benzoyl- $\alpha$ -aminocinnamate I melting at 135–145°. The benzene solution was concentrated, giving an oil which was dissolved in 30 cc. of alcohol. The solution was cooled in the ice box, giving an additional 4 gm. of Ester I. The filtrate was concentrated to an oil which slowly crystallized, giving impure ethyl benzoyl- $\alpha$ -aminocinnamate II melting at 70–80°. Recrystallization of this material from ethyl alcohol yielded a still impure Ester II, melting at 75–88°. Since considerable material is lost in recrystallization, crude Ester II melting at 70–80° was used for subsequent reactions.

*Benzoyl- $\alpha$ -Aminocinnamic Acids I and II*—10 gm. of ethyl benzoyl- $\alpha$ -aminocinnamate I were suspended in 300 cc. of alcohol and 200 cc. of 0.5 N sodium hydroxide were added. The mixture was allowed to stand at room temperature with occasional shaking for 2 hours. (Most of the solid had disappeared in 30 to 40 minutes.) The solution was diluted with 200 cc. of water and extracted twice with ether. The aqueous layer was filtered and acidified, giving 7.5 gm. (83 per cent) of benzoyl- $\alpha$ -aminocinnamic acid I melting at 223–226°.

Benzoyl- $\alpha$ -aminocinnamic acid azlactones I and II may be hydrolyzed by the same procedure, thus affording an improved method of converting the azlactones to acids in one step.

8 gm. of crude ethyl benzoyl- $\alpha$ -aminocinnamate II melting at 70–80° were hydrolyzed by the above procedure, giving 6.2 gm. (85 per cent) of a mixture of benzoyl- $\alpha$ -aminocinnamic acids melting at 180–190°. This material was extracted with 35 cc. of alcohol. The residue (4 gm., melting at 190–193°) was dissolved in a mixture of 200 cc. of benzene and 20 cc. of absolute alcohol. 200 cc. of petroleum ether were added and the mixture was cooled overnight in the ice box, giving 3.4 gm. of material melting at 196–198°. A second recrystallization gave 3.1 gm. of pure benzoyl- $\alpha$ -aminocinnamic acid II melting at 199–200°.

$C_{16}H_{13}O_3N$ . Calculated.	N 5.24,	neutral equivalent	267
Found.	" 5.12,	"	265

A mixture of benzoyl- $\alpha$ -aminocinnamic acids I and II melted at 175–190°.

*Benzoyl- $\alpha$ -Aminocinnamic Acid Azlactones I and II*—1.0 gm. of benzoyl- $\alpha$ -aminocinnamic acid I was heated on the steam cone with 10 cc. of acetic anhydride for 2 minutes. The solution was

poured onto a mixture of ice and water and allowed to stand with occasional stirring for 20 minutes. The solid was removed by filtration, air-dried, and recrystallized from benzene-alcohol, giving 0.8 gm. of Azlactone I melting at 164–166°.

2.0 gm. of benzoyl- $\alpha$ -aminocinnamic acid II were treated in the same manner. The air-dried reaction product was dissolved in 8 cc. of warm benzene and 14 cc. of absolute alcohol were added. The solution was cooled overnight in the ice box, yielding 1.2 gm. of benzoyl- $\alpha$ -aminocinnamic acid azlactone II melting at 146–148°. A mixture of equal parts of this material and Azlactone I melted at 125–135°.

$C_{16}H_{11}O_2N$ . Calculated, N 5.62; found, N 5.60

0.3 gm. of benzoyl- $\alpha$ -aminocinnamic acid azlactone II was dissolved in 3 cc. of pyridine at room temperature. After 3 minutes the solution was poured into an excess of iced hydrochloric acid. The precipitate consisted of practically pure Azlactone I melting at 163–165°.

#### *$\beta$ -Phenethylamine Salts*

$\beta$ -Phenethylamine was prepared by reducing benzyl cyanide with a Raney nickel catalyst. We confirmed the report (7) that the use of liquid ammonia as a solvent increases the yield of  $\beta$ -phenethylamine to 80 to 90 per cent.

Benzoyl-*dl*-O-methylphenylserine B, crude benzoyl-*dl*-O-methylphenylserine A (melting at 152–154°), N-benzoyl-*dl*-threonine, N-benzoyl-*dl*-allothreonine, benzoyl-*dl*-O-methylthreonine, and benzoyl-*dl*-O-methylallothreonine were prepared as described previously (2, 8). Carbobenzoxy-*dl*-O-methylphenylserine B was prepared from the pure Amino Acid B in the usual manner (9). The reaction product was recrystallized from 10 volumes of hot benzene, giving the pure carbobenzoxy derivative melting at 140–142°.

$C_{18}H_{19}O_5N$ . Calculated. N 4.26, neutral equivalent 329  
Found. " 4.18, " " 331

Carbobenzoxy-*dl*-O-methylphenylserine A was prepared from crude Amino Acid A. The product was an oil which slowly solidified to an amorphous glass. This material could not be induced to crystallize. It was therefore purified through the  $\beta$ -phenethyl-

amine salt. The regenerated carbobenzoxy derivative crystallized readily. It was recrystallized from 1.5 volumes of benzene, giving pure carbobenzoxy-*dl*-O-methylphenylserine A melting at 103-105°.

$C_{18}H_{19}O_5N$ . Calculated. N 4.26, neutral equivalent 329  
Found. " 4.20, " " 327

The  $\beta$ -phenethylamine salts were prepared by dissolving or suspending the derivative in 10 to 30 volumes of warm ethyl acetate and adding the calculated quantity of amine. In the case of insoluble derivatives the reaction mixture was warmed and stirred for several minutes. The salts usually crystallized, either im-

TABLE I  
 *$\beta$ -Phenethylamine Salts*

$\beta$ -Phenethylamine salt	M.p.	Formula	N analyses	
			Calculated	Found
	°C.		per cent	per cent
<i>dl</i> -O-Methylphenylserine				
Benzoyl A.....	184-188	$C_{25}H_{23}O_4N_2$	6.67	6.48
" B.....	169-171	"	6.67	6.52
Carbобензоxy A.....	132-135	$C_{26}H_{25}O_5N_2$	6.22	6.13
" B.....	80- 86	"	6.22	6.28
N-Benzoyl- <i>dl</i> -allothreonine.....	148-152	$C_{19}H_{24}O_4N_2$	8.14	7.87
N-Benzoyl- <i>dl</i> -threonine.....	159-162	"	8.14	7.86
N-Benzoyl- <i>dl</i> -O-methylthreonine...	113-117	$C_{20}H_{26}O_4N_2$	7.82	7.72
N-Benzoyl- <i>dl</i> -O-methylallothreonine ..	126-130	"	7.82	7.57

mediately, or on cooling when the walls of the flask were scratched. In the case of benzoyl- and carbobenzoxy-*dl*-O-methylphenylserine B it was necessary to concentrate the solution considerably before the salts separated. The salts were recrystallized from ethyl acetate or from an alcohol-ethyl acetate mixture in the case of the less soluble salts. The melting points and analyses of the salts are summarized in Table I.

The  $\beta$ -phenethylamine salts were decomposed by dissolving or suspending them in dilute ammonium hydroxide and acidifying the mixture with concentrated hydrochloric acid. By purification in this manner benzoyl-*dl*-O-methylphenylserine A, melting sharply at 166-167°, was obtained, and carbobenzoxy-*dl*-O-methylphenyl-

serine A was prepared in a pure crystalline state. The latter derivative was reduced in the usual manner (9), giving *dl*-O-methylphenylserine A melting at 231–233°. Benzoylation of this material gave Benzoyl Derivative A melting at 165–167°.

## SUMMARY

1. Two benzoyl- $\alpha$ -aminocinnamic acid azlactones are produced by the action of acetic anhydride on either of the benzoyl-*dl*-O-methylphenylserines. Certain reactions of the azlactones are described.

2. A study has been made of the  $\beta$ -phenethylamine salts of the following pairs of isomers: benzoyl-*dl*-O-methylphenylserines A and B; carbobenzoxo-*dl*-O-methylphenylserines A and B; N-benzoyl-threonine and N-benzoylallothreonine; benzoyl-*dl*-O-methylthreonine and benzoyl-*dl*-O-methylallothreonine. In each pair the lower melting, more soluble derivative gives the higher melting, less soluble  $\beta$ -phenethylamine salt. It seems possible, therefore, that  $\beta$ -phenethylamine may be generally useful in separating isomers of this type.

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## THE VERATRINE ALKALOIDS

### VIII. FURTHER STUDIES ON THE SELENIUM DEHYDROGENATION OF CEVINE

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In a previous publication (1) we have reported a confirmation at least in part of the results obtained by Blount (2) in the dehydrogenation of cevine with selenium as well as the isolation of several other products also formed during the reaction. The products isolated other than the simpler pyridine derivatives are unique in character and undoubtedly will play an important rôle in the final elucidation of the structure of the veratrine alkaloids. Unfortunately, the yields of these products are rather small because of the complicated course of the decomposition of the alkaloid and they are difficult to isolate owing undoubtedly to the formation of a number of closely related products the properties of which are very similar.

More recently we have turned again to this study with the intention of making a more thorough examination of the reaction mixture in the hope of effecting a more complete separation leading to other products the study of which might aid in the interpretation of the structure of those already isolated and further of increasing the supply needed for chemical investigation. Accordingly we have dehydrogenated a larger amount of cevine for the purpose. The dehydrogenation was carried out as previously reported except that the temperature was raised to 345°.

The products from all of the runs were combined and processed together. The general procedure was to effect a separation first into basic, neutral, and phenolic fractions, after which each fraction was subjected to chromatographic analysis in a chromatograph of the appropriate size prepared from Brockmann's alumina. In

only one case did any of the fractions from the chromatographic separation appear to approach homogeneity, although the method served to give a separation into classes of substances which had approximately the same affinity for the adsorbent and were probably for the most part a mixture of homologues. Each of the fractions coming from the chromatograph was then subjected to microfractional distillation analysis with the use of the apparatus and technique indicated in a previous publication (3). The cevanthridine fraction proved to have too high a boiling point for efficient separation without the danger of pyrolysis in this type of column. Also many of the fractions from the chromatograph yielded less than 0.4 gm. of substance, an amount too small for efficient fractionation.

Fractional distillation served to separate the chromatographic fractions into different molecular sizes and frequently separated more or less completely mixtures of homologues in which the operating conditions were favorable to high efficiency of the column. Usually, however, it was necessary to study further the fractions from the fractional distillation by means of microfractional crystallization as well as by the preparation of derivatives.

This general method of procedure has resulted in the isolation of fifteen degradation products, the formulations of which appear to be reasonably certain, as well as of others in which homogeneity is still in question. The majority of these products have not as yet been reported. The isolation of the following list of substances which includes those already described is therefore reported in this paper:  $\beta$ -picoline  $C_6H_7N$ , 2-ethyl-5-methylpyridine  $C_8H_{11}N$ , base  $C_9H_{13}N$ , 2-hydroxyethyl-5-methylpyridine  $C_8H_{11}ON$ , oxyethylmethylpyridine (cyclic ether?)  $C_8H_9ON$ , hydrocarbon (4,5-benzohydrindene)  $C_{13}H_{12}$ , hydrocarbon  $C_{17}H_{16}$ , hydrocarbon  $C_{18}H_{18}$ , hydrocarbon  $C_{19}H_{20}$ , hydrocarbon  $C_{24}H_{30}$ , cevanthrol  $C_{17}H_{16}O$ , an oxygen-containing substance  $C_{23}H_{24}O$ , cevanthridine  $C_{25}H_{27}N$ ,<sup>1</sup> base  $C_{20}H_{19}N$ , base  $C_{26}H_{25}N$ .

The relationships of the first five of these substances seem to be quite clear on the basis of the structures already discussed (1). Both the third and fourth substances, which have not been re-

<sup>1</sup> In a succeeding paper, evidence for the formulation of this substance will be presented which seems to favor rather the formula  $C_{25}H_{27}N$  than that of  $C_{25}H_{25}N$  originally proposed by Blount (2).

ported before from the selenium dehydrogenation, are entirely compatible. Concerning the nature of the remaining degradation products considerable information is now at hand from a study of absorption spectra as well as of certain chemical transformations. This will be discussed in the succeeding papers. As will be seen, the hydrocarbon  $C_{18}H_{12}$  has been found to correspond in properties with those of 4,5-benzohydrindene.

#### EXPERIMENTAL

120 gm. of recrystallized cevine were dehydrogenated in six separate runs as previously described (1) except that the temperature employed was  $345^{\circ}$ . The volatile material in the ice trap was removed and treated with a slight excess of HCl. The mixture was extracted with ether. After concentration of the ether extract, the resulting residual thick oil (about 1.5 gm.) was investigated by chromatographic analysis followed by fractional distillation in accordance with procedures essentially the same as those used with the major reaction mixture as described below. All that could be obtained from such non-basic material carried over into the ice trap was a series of oils. From these again the only crystalline material that could be isolated appeared to be the  $C_{18}H_{18}$  hydrocarbon previously reported. Although the amount of material available was too meager for exhaustive recrystallization, the micro melting point finally reached  $104-107^{\circ}$  and showed no depression with a more extensively recrystallized sample of other origin which melted at  $116-118^{\circ}$ .

$C_{18}H_{18}$ . Calculated, C 92.24, H 7.77; found, C 92.12, H 7.65

Analytical data indicated the various oily fractions to be cyclic hydrocarbons in a partial state of dehydrogenation.

The HCl solution which remained after the above ether extraction was treated with excess solid KOH and the basic material which separated was extracted with benzene. The benzene extract was dried over anhydrous  $K_2CO_3$  and run through a chromatograph prepared with benzene and 400 gm. of Brockmann's alumina. As soon as material other than solvent appeared, a volume of 400 cc. was collected. Further fractionation of the material in the chromatograph is described below. This was fractionated first through a Vigreux column to remove most of



the benzene. The residual oil was then placed in a small column having a separating power of roughly ten theoretical plates as measured by mixtures of carbon tetrachloride-benzene. After the residual benzene had distilled, a fraction boiling at 69–75° and under 23 mm. pressure was collected. The volume of this fraction was approximately 1.5 cc. It was presumably a mixture of the previously reported  $\beta$ -picoline and 2-ethyl-5-methylpyridine, since it had a micro boiling point of 165° at atmospheric pressure and the analytical data proved to be intermediate between the figures calculated for  $C_6H_7N$  and  $C_8H_{11}N$ .

$C_6H_7N$ . Calculated, C 77.37, H 7.58

$C_8H_{11}N$ . " " 79.27, " 9.15; found, C 78.64, H 8.62

TABLE I

Fraction No.	Oil bath temperature	Pressure	Weight	Micro b.p.	Analysis	
					C	H
	°C.	mm.	mg.	°C.	per cent	per cent
1	106	8	70	187	79.61	9.17
2	106	5	70	189	79.59	9.76
3	106	2	70	199	77.35	9.02
4	110	0.6	70	210	73.84	7.95
5	150	0.1	60	216	78.75	9.35
6	200	0.1	100	260	77.80	9.13
7 Approximately 200 mg. of residue removed from still						

The next fraction was of more constant boiling point and amounted to approximately 1 cc. It had a micro boiling point of 173° at atmospheric pressure and gave the correct analytical figures for ethylmethylpyridine.

Found. C 79.05, H 9.05

The residue in the still which was less than 1 cc. in volume was too small in amount for the column in use and was therefore transferred to a column of the type described previously (3). This column was 5 cm. in height and had a separating power in the neighborhood of eight theoretical plates. The data for the fractionation are given in Table I.

Each of the fractions was investigated carefully for the presence

of a volatile quinoline derivative which, if it had been formed, should have appeared in one of these fractions. However, we were unable to locate such a derivative and our investigation therefore seems to eliminate the possibility of the production of a simple quinoline derivative. The analytical data also indicate nothing higher in carbon or lower in hydrogen than an alkyl-substituted pyridine or oxypyridine.

*The Base  $C_9H_{13}N$* —Fraction 2 gave analytical data suggesting a formula  $C_9H_{13}N$ .

$C_9H_{13}N$ . Calculated, C 79.93, H 9.70; found, C 79.59, H 9.76

It gave a picrate that crystallized from alcohol in broad thin leaves which melted at 150–151°.

$C_9H_{13}N \cdot C_6H_3O_7N_3$ . Calculated, C 49.43, H 4.43; found, C 49.30, H 4.20

This picrate as well as the free base corresponded with the properties of the pyridine base,  $C_9H_{13}N$ , previously obtained from the zinc dust distillation of cevine (4). A mixed melting point of the picrates showed no depression. Since in the previous publication it was shown that the base upon oxidation with  $KMnO_4$  yielded a pyridinedicarboxylic acid possessing one more  $CH_2$  group than isocinchomeric acid, it is perhaps a homologue of ethylmethylpyridine and has the additional  $CH_2$  group in a position which prevents or retards its oxidation.

*The Base  $C_8H_9ON$* —Fraction 4 gave analytical figures indicating the presence of an oxygen-containing base, although it was evidently still a mixture. 20 mg. of the base were treated with an equivalent of picric acid and the resulting picrate was crystallized from acetone. 25 mg. of heavy rhombs were obtained which melted at 150–151°. After recrystallization, the melting point was 151–152°. This melting point agreed with that previously reported for the picrate of the base  $C_8H_9ON$  (1).

$C_8H_9ON \cdot C_6H_3O_7N_3$ . Calculated, C 46.14, H 3.30; found, C 46.31, H 3.31

*Isolation of the Base  $C_8H_{11}ON$* —After collection of the first 400 cc. of eluent from the chromatograph above a second volume of 500 cc. of benzene was collected. Upon fractionation approximately 0.3 gm. of an oil was obtained which appeared mostly to boil in the region of  $\beta$ -picoline and was not further studied.

A liter of ether was then run through the chromatograph. Similar fractionation of the resulting oil (0.4 gm.) also yielded for the most part fractions approaching the boiling point of  $\beta$ -picoline.

500 cc. of methyl alcohol were then run through the chromatograph. Fractionation of this gave, after removal of the solvent, 2 cc. of water and then 1.3 cc. of a viscous higher boiling oil. The latter fraction was placed in a 22 cm. microfractionating column (3). The data for the fractionation are given in Table II.

After Fraction 3 the micro boiling point was fairly constant and since Fractions 2, 7, and 10 all gave similar analytical data, it appeared likely that they were all the same and consisted of a

TABLE II

Fraction No.	Bath temperature	Column temperature	Pressure	Weight of fraction	Micro b.p.	Analysis	
						C	H
	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	<i>mm.</i>	<i>mg.</i>	$^{\circ}\text{C.}$	<i>per cent</i>	<i>per cent</i>
1	102	90	5	20	215	69.82	7.78
2	102	90	5	40	225		
3	102	100	5	80	227		
4	102	100	5	80	229		
5	102	95	4	80	229		
6	102	92	4	80	229	69.93	8.24
7	102	92	4	100	229		
8	102	92	4	70	229		
9	105	92	4	80	229	69.83	8.18
10	110	98	4	80	229		
11	115	105	4	80	229		

substance with the empirical formula of a hydroxyl derivative of ethylmethylpyridine.

$\text{C}_8\text{H}_{11}\text{ON}$ . Calculated, C 70.02, H 8.08

The picrate could not be induced to crystallize. It gave no color with ferric chloride and could not be methylated with diazomethane. The substance did not have phenolic properties, since it was extracted with benzene in the earlier isolation from a strong solution of KOH. The hydroxyl group appears therefore to be on the side chain. This is supported by the oxidation of the substance with  $\text{KMnO}_4$  to isocinchomeronic acid as follows:

50 mg. of the base were dissolved in 10 cc. of water and treated

with 350 mg. of  $\text{KMnO}_4$ . After heating on the steam bath for 6 hours, the  $\text{MnO}_2$  was filtered off. The clear filtrate was treated with normal  $\text{HCl}$  until just acid to Congo red and then 0.5 cc. in excess was added. After concentration to about 1 cc. and chilling, 25 mg. of needles were collected which melted at  $260\text{--}261^\circ$ , depending somewhat on the rate of heating. A mixed melting point with material obtained from the oxidation of 2-ethyl-5-methylpyridine (5) showed no depression.

$\text{C}_7\text{H}_5\text{O}_4\text{N}$ . Calculated, C 50.30, H 2.99; found, C 50.65, H 3.08

*Isolation of Hydrocarbons*—The residue which remained in the original dehydrogenation flask was ground up and exhaustively extracted with ether. The ether was shaken out with 10 per cent  $\text{HCl}$ . The lower acid layer contained much solid insoluble hydrochloride and was set aside to be treated as described below for the basic part. The dried ether extract gave approximately 7 gm. of residue which was dissolved in 100 cc. of benzene. This solution was run through a chromatograph prepared with 500 gm. of Brockmann's alumina suspended in benzene. As soon as dissolved material began to appear at the lower end, each 100 cc. quantity of eluent was considered a fraction and was evaporated to dryness and weighed. The weights were as follows: Fraction 1, 1.600 gm., oil; Fraction 2, 0.560 gm., oil; Fraction 3, 0.200 gm., oil; Fraction 4, 0.115 gm., oil; Fraction 5, 0.120 gm., oil; Fraction 6, 0.106 gm., oil; Fraction 7, 0.085 gm., oil; Fraction 8, 0.085 gm., oil; Fraction 9, 0.040 gm., oil; Fraction 10, 0.040 gm., partially crystalline; Fraction 11, 0.030 gm., partially crystalline; Fraction 12, 0.110 gm., partially crystalline (from 500 cc. of eluent).

1 liter of anhydrous ether was then run through the chromatograph. This eluted 200 mg. more of material which could not be induced to crystallize. 400 cc. of methyl alcohol were then employed. Upon evaporation this extract proved to contain 2.7 gm. of material which was oxygen-containing and which contained the cevanthrol fraction. Our investigation of this fraction thus far has yielded only cevanthrol (6), although the major portion of the fraction appears to consist of material giving similar analytical data but to have a lower, indefinite melting point. It is probably composed of substances closely related to cevanthrol and with similar properties.

The above Fractions 10, 11, and 12 eluted with benzene yielded partly crystalline residues which appeared to have similar properties and were therefore combined. After sublimation in a high vacuum and two recrystallizations from ether a small amount of material was finally obtained which gave a micro melting point of 181–187°. This material was insufficient for both recrystallization and analysis. The latter indicated a formula of  $C_{23}H_{24}O$ .

$C_{23}H_{24}O$ . Calculated, C 87.29, H 7.65; found, C 87.21, H 7.77

Fractions 1 and 2 from above were combined and placed in a sublimation apparatus. All was collected which distilled up to

TABLE III

Fraction No.	Weight	Oil bath temperature	Column temperature	B.p. at 760 mm.	Micro m.p.	Analysis	
						C	H
	mg.	°C.	°C.	°C.	°C.	per cent	per cent
1	100	120	70	80			
2	50	160	90	266		90.15	9.27
3	70	170	108	293		90.68	8.73
4	60	180	130	320		90.63	9.06
5	80	185	145	350		90.16	9.21
6	130	190	160		Up to 157	91.67	8.50
7	120	195	160		" " 150	91.75	8.34
8	130	205	165		" " 99	91.99	8.31
9	160	210	165		" " 110	91.85	8.26
10	120	215	175		" " 178	91.70	8.55
11	135	225	195		" " 177	91.80	8.11
12	170	235	210		" " 85	91.07	8.62
13	135	235	215		" " 102	90.74	9.32

an oil bath temperature of 200° and under a pressure of 0.25 mm. The distillate weighed 1.8 gm. This oil was placed in a 22 cm. column (3). The data for the fractionation which was carried out under a pressure of 0.25 mm. are given in Table III.

Although the analytical data did not shift much from fraction to fraction, a closer examination by fractional recrystallization revealed satisfactory separation into different molecular sizes. The original material was apparently composed of mixtures of homologues in various stages of dehydrogenation.

From the boiling point it is obvious that Fraction 1 (Table III) is benzene. Fractions 2 and 3 both yielded crystalline picrates.

Since the picrates appeared to have the same properties, they were combined. Thus approximately 70 mg. of a picrate were obtained which melted at 90–100° (micro melting point). After recrystallization from alcohol heavy orange needles were obtained which melted at 99–103°. This material gave analytical figures which corresponded to a hydrocarbon of the formula  $C_{13}H_{12}$ .

$C_{13}H_{12} \cdot C_6H_5O_7N_3$ . Calculated, C 57.43, H 3.80; found, C 57.77, H 3.62

45 mg. of the picrate were dissolved in ether and the picric acid was removed with 2 per cent NaOH. The recovered hydrocarbon weighed 21 mg. and formed a colorless oil. It was redistilled under reduced pressure. The colorless distillate had only a faint odor and could not be induced to crystallize.

$C_{13}H_{12}$ . Calculated, C 92.86, H 7.20; found, C 92.85, H 6.88

A few mg. of the above picrate were recrystallized from ethyl alcohol. The substance had a micro melting point of 103–105°. After a further recrystallization the stout orange needles melted at 106–107°. Since there was insufficient for further recrystallization, this material was used for the comparison with synthetic 4,5-benzohydrindene kindly placed at our disposal by Professor J. W. Cook of the University of Glasgow. This comparison will be discussed in the following paper.

Fractions 4 and 5 (Table III) failed to give crystalline picrates.

*The  $C_{17}H_{16}$  Hydrocarbon*—Fraction 6 which was almost entirely crystalline was recrystallized from ether. 35 mg. of material were obtained with a micro melting point of 147–157°. A further recrystallization gave 19 mg. of thin leaves which melted at 160–165°.

$C_{17}H_{16}$ . Calculated, C 92.68, H 7.32; found, C 92.93, H 7.25

The molecular weight determination was made by the Rast method.

0.592 mg. substance : 6.042 mg. camphor;  $\Delta = 16.0^\circ$

Mol. wt. found, 222; calculated, 220.13

After a further recrystallization the melting point was 167–169°.

The picrate dissociated very easily but could be crystallized in orange needles from a concentrated solution in benzene. The

micro melting point was 127–129° but the analytical data indicated a much larger proportion of hydrocarbon than is required by the 1:1 molecular ratio.

This hydrocarbon corresponded to the same material for which the formula  $C_{17}H_{16}$  was derived previously (1), although the melting point now obtained was considerably higher obviously owing to greater purity.

Fraction 7 after recrystallization in the same manner as in the case of Fraction 6 gave a similar amount of material that showed no depression in a mixed melting point with that from Fraction 6. It was therefore presumably the same substance.

*The  $C_{18}H_{18}$  Hydrocarbon*—Fraction 9 after recrystallization from ether gave 55 mg. with a micro melting point of 85–110°. Upon recrystallization 30 mg. of thin leaves resulted which melted at 109–114°.

$C_{18}H_{18}$ . Calculated, C 92.24, H 7.77; found, C 92.28, H 7.65

A portion of this material after recrystallization from ether showed a micro melting point of 114–116° and was identical with the  $C_{18}H_{18}$  hydrocarbon previously reported (1). The picrate of this substance also dissociated very easily and was not suitable for characterization.

Fraction 8 behaved similarly upon recrystallization and showed no depression in mixed melting point with the substance from Fraction 9. However, a mixed melting point with the hydrocarbon from Fraction 6 was 102–136°.

*The Hydrocarbon  $C_{19}H_{20}$* —Fraction 11 was recrystallized from ether. This yielded 50 mg. with a micro melting point of 140–162°. After recrystallization from ether again 24 mg. of broad glistening leaves were obtained which melted at 173–182°.

$C_{19}H_{20}$ . Calculated, C 91.88, H 8.12; found, C 91.77, H 8.27

The molecular weight determination was made by the Rast method.

0.583 mg. substance : 6.020 mg. camphor;  $\Delta = 14.0^\circ$

Mol. wt. found, 249; calculated, 248.16

The above material after recrystallization from ether melted at 185–188°.

Fraction 10 behaved like Fraction 11 and a mixed melting point showed no depression. However, this material showed a definite depression when mixed with either that from Fraction 6 or 8.

*The Hydrocarbon  $C_{24}H_{30}$* —Fraction 12 contained much finely divided selenium, although the other fractions were free from this impurity. Apparently the small amount of residual selenium was fractionated out at this point. After removal of the selenium the hydrocarbons remaining proved to be too much of a mixture for successful separation.

Fraction 13 upon recrystallization from ether gave 50 mg. with a micro melting point of 97–106°. After recrystallization broad thin leaves were obtained which melted at 106–109°.

$C_{24}H_{30}$ . Calculated, C 90.51, H 9.50; found, C 90.45, H 9.64

The molecular weight determination was made by the Rast method.

0.913 mg. substance : 9.230 mg. camphor;  $\Delta = 11.1^\circ$   
Mol. wt. found, 320; calculated, 318.24

A further recrystallization yielded material melting at 108–110°.

The aqueous acid layer which remained after removal of the ether solution from the original reaction mixture contained a considerable amount of insoluble precipitate which proved to be the cevanthridine fraction. The HCl solution which was separated from the solid was found to contain only simpler pyridine bases. The solid material was dissolved in chloroform and shaken out with 10 per cent KOH. After the solution was dried over  $K_2CO_3$ , the solvent was removed *in vacuo*. The residue was dissolved in benzene and the evaporation repeated in order to remove chloroform. The residue which weighed 25 gm. was redissolved in 100 cc. of benzene and run through a chromatograph prepared with 1.5 kilos of Brockmann's alumina suspended in benzene. As soon as material began to emerge with the solvent each succeeding volume of 150 cc. was considered a fraction. Fractions 1 to 5 contained only oils that could not be induced to crystallize but which after distillation in a high vacuum gave analytical results closely approximating the values for cevanthridine. Fractions 6 to 12 were all nearly entirely crystalline and constituted the cevanthridine fraction. This combined fraction weighed 5 gm.



and yielded 1 gm. of pure cevanthridine upon recrystallization. A discussion of the analysis, etc., of this substance will be left to a following paper.

The succeeding chromatograph fractions appeared to yield material of a different character. The materials in Fractions 22, 23 and 24 appeared to be the same and were combined. Upon recrystallization a substance was obtained corresponding in properties with that previously reported (1) and melted at 229–230°. However, the formula  $C_{26}H_{25}N$  now appears more probable than that of  $C_{25}H_{25}N$  originally proposed.

$C_{26}H_{25}N$ .	Calculated.	C 88.84, H 7.17, N 3.99
	Found.	" 88.77, " 7.25, " 4.35
		" 88.87, " 7.02, " 4.13
		" 89.16, " 7.08, " 3.97

*Methiodide of the Base  $C_{26}H_{25}N$* —20 mg. of the base were treated with 5 cc. of methyl iodide and allowed to stand several hours. The collected product after recrystallization from a large volume of methyl alcohol formed fine needles which melted with decomposition at about 295°, depending somewhat on the rate of heating.

$C_{27}H_{23}NI$ .	Calculated, C 65.71, H 5.71; found, C 65.75, H 5.77
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Little material was contained in Fraction 30 from the chromatograph, so the benzene was replaced by anhydrous ether as eluent. Fractions 1 to 4 obtained with ether did not contain material that would crystallize but Fractions 5 to 10 all showed a tendency to crystallize. They were therefore combined and crystallized from ether. 225 mg. of material were obtained which was recrystallized twice from benzene and then from chloroform and melted at 226–231°. A final recrystallization from chloroform with bone-black gave 92 mg. of broad, thin leaves which melted at 233–235°.

$C_{20}H_{19}N$ .	Calculated.	C 87.88, H 7.01, N 5.12
	Found.	" 88.06, " 7.25, " 5.15

This substance added methyl iodide as in the case of cevanthridine, and the analysis of the methiodide supported the formulation above. It crystallized from methyl iodide in yellow leaves which

decomposed at 285–290°, depending somewhat on the rate of heating.

$C_{21}H_{22}NI$ . Calculated, C 60.71, H 5.34; found, C 60.86, H 5.41

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## THE VERATRINE ALKALOIDS

### IX. THE NATURE OF THE HYDROCARBONS FROM THE DEHYDROGENATION OF CEVINE

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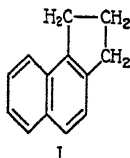
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In the previous paper (1) we have reported the isolation of a series of hydrocarbons from the selenium dehydrogenation of cevine. Since the amount of each substance isolated in a form approaching purity was too small for extended investigation by chemical transformation, our attention has been turned to a study of their absorption spectra. Considerable information is now at hand in the literature regarding the types of absorption spectra in the region of the ultraviolet which are more or less characteristic of the various aromatic ring systems. The formulations of our unknown hydrocarbons as well as their general nature have made them therefore particularly interesting for a study from this standpoint. The results of these investigations are reported in this paper.<sup>1</sup>

The simplest hydrocarbon of the series appeared to possess the formula  $C_{13}H_{12}$  derived by analysis of the hydrocarbon itself and of its picrate. The number of carbon and hydrogen atoms in this formulation at once places limitations on the possible ring systems which can be considered. A number of combinations of a benzene ring attached to unsaturated 5-membered rings such as indene might be considered but such unsaturated systems could scarcely be expected to withstand the conditions of dehydrogenation. Only a naphthalene ring system with a saturated ring attached can be seriously considered. The possibilities in this

<sup>1</sup> The absorption curves were obtained with a Spekker spectrophotometer and a small Hilger quartz spectrograph. The solvent in each case was absolute alcohol.

category are 4,5-benzohydrindene, 5,6-benzohydrindene, perinaphthane, and one of the four possible methylacenaphthenes. Perinaphthane melts at  $60^{\circ}$  and gives a picrate melting at  $160^{\circ}$  (2), whereas 5,6-benzohydrindene melts at  $94^{\circ}$  and yields a picrate melting at  $120^{\circ}$  (3). 4,5-Benzohydrindene (4) is an oil and its picrate melts at  $109\text{--}110^{\circ}$ . Our substance was likewise an oil but the small amount of substance available because of the very tedious process involved in its isolation did not permit recrystallization of its picrate to a constant melting point. The melting point, however, on the final recrystallization changed only from  $103\text{--}105^{\circ}$  to  $106\text{--}107^{\circ}$ . It therefore appeared to approach closely the properties of 4,5-benzohydrindene (Formula I). Further



identity now appears to have been definitely established by a direct comparison with synthetic material which was very kindly placed at our disposal by Professor J. W. Cook of the University of Glasgow. A mixed melting point of the picrates from both sources did not show an appreciable depression and the two substances appeared identical in all their properties. Comparison of the ultraviolet absorption spectrum of synthetic 4,5-benzohydrindene with that of our oil (Fig. 1) with a few minor exceptions showed a close agreement and gave further strong support to the question of identity.

The next hydrocarbon of the series isolated appears from the analysis to possess the formula  $C_{17}H_{16}$ . Empirically this formulation allows for a total of ten double bonds plus rings and corresponds to a trimethylphenanthrene or an anthracene. However, its ultraviolet absorption spectrum curve is quite different from that of either anthracene or phenanthrene (Fig. 2). The difference appears to be sufficiently great to eliminate the double bond arrangement of either of these ring systems from serious consideration.

The formulation of the next three hydrocarbons,  $C_{18}H_{18}$ ,  $C_{19}H_{20}$ ,

and  $C_{24}H_{30}$  respectively, also implies a total of ten double bonds plus rings and this fact, along with their common origin, suggests a close relationship in the ring structure of the four hydrocarbons. The striking similarity of their absorption spectra as shown in Fig. 3 makes this seem even more evident.

Although the type of their absorption spectra seems to be unrelated to either the phenanthrene or the anthracene type, it appears to approach more closely the general type given by naph

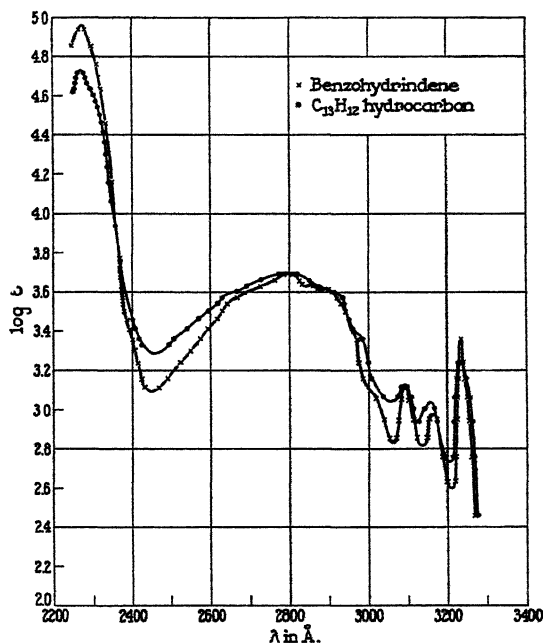


FIG. 1

thalene derivatives. Accordingly, a naphthalene ring system to which are joined three other rings or double bonds might be considered as a possibility. Since a cyclopentenonaphthalene ring system in the hydrocarbon  $C_{13}H_{12}$  has been isolated from the same dehydrogenation mixture, it could be suggested that these hydrocarbons contain such a ring system to which either two additional rings are attached or one ring containing a double bond. Of these two possibilities the latter might appear to be more definitely

suggested by the absorption spectra on the naphthalene basis. These show a greater absorption coefficient in general than do naphthalene derivatives which do not carry a group containing a conjugated double bond.

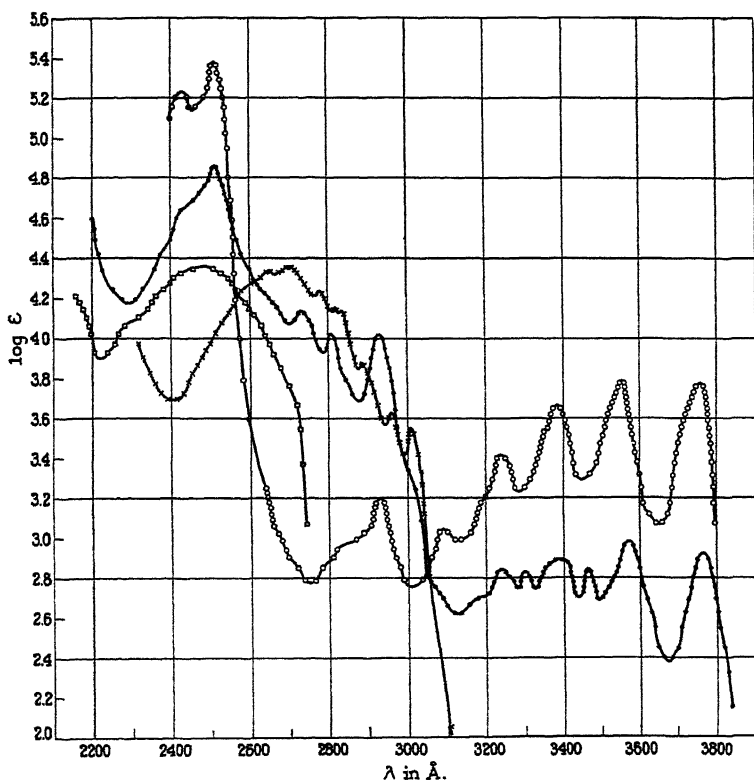


FIG. 2.  $\times$  =  $C_{17}H_{16}$  hydrocarbon;  $\bullet$  = phenanthrene;  $\circ$  = anthracene;  $\square$  = diphenyl.

It is improbable from general experience that such an extra unsaturated ring could be 5-membered. Should an extra 6-membered ring be attached to the naphthalene ring system in any positions other than the 1,8 or peri positions, the hydrocarbon would then be a tetrahydroanthracene or phenanthrene derivative and under the conditions used for the dehydrogenation from which it was isolated, such a derivative might be expected to be dehydro-

generated to its parent phenanthrene or anthracene. While ordinarily failure to isolate such a derivative from a very complex mixture cannot be considered as final evidence against its presence there, it should be pointed out that a very thorough search was made which had resulted in the isolation of some fifteen substances among which are five hydrocarbons having almost the identical properties which such derivatives would possess. Nevertheless,

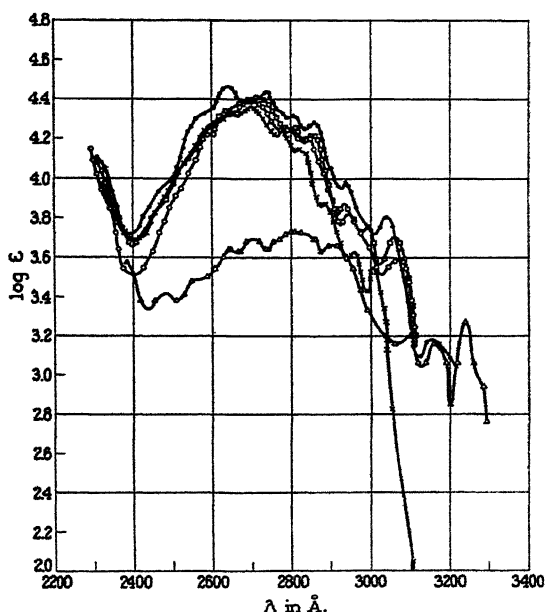


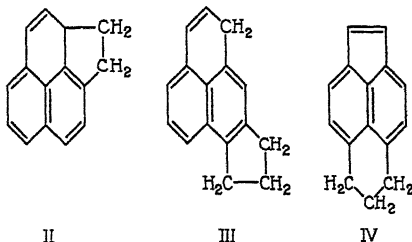
FIG. 3.  $\Delta$  =  $C_{13}H_{12}$ ;  $\times$  =  $C_{17}H_{16}$ ;  $\circ$  =  $C_{18}H_{18}$ ;  $\bullet$  =  $C_{19}H_{20}$ ;  $\square$  =  $C_{24}H_{20}$

all attempts to isolate tetrahydrophenanthrenes or anthracenes have failed.

This evidence should be considered together with the fact that there may be present in the alkaloid a hydrogenated naphthalene ring system which is substituted in the 1,8 positions, if our deductions (5) regarding the general structure of decevinic acid and the assumption of its primary character are correct. Thus the possibility must be considered that the more complex hydrocarbons may contain the cyclopentenonaphthalene ring system to which a further ring is joined at the peri positions. In accordance with



this, there may be suggested two structures which are derivatives of the little studied benzonaphthene ring system, Formulas II and III, and a third structure, Formula IV. In each case a number of modifications are of course conceivable on the basis of different arrangements of the double bonds.



From the work of Pestemer and Manchén (6) the approximate effect on the absorption spectrum of a double bond conjugated with the naphthalene ring system can be seen. The absorption coefficient is increased considerably and the bands are shifted toward the longer wave-lengths, an effect in conformity with past experience for the conjugation of a double bond with an aromatic nucleus. The opposite of this last effect has been noted in the case of our substances, if referred to naphthalene.

The question might persist as to whether the reversed shift noted with our substances could be due to any one if not all of the possible arrangements of rings and double bonds in the benzonaphthene ring system.

Since the synthesis of any of the ring structures represented above is a major research in itself and since hydrocarbons with this ring structure appear to be little if at all investigated, it seemed advisable to turn to simpler known substances in order to see what arrangement of double bonds might be indicated in our hydrocarbons by the study of the absorption spectra of such model substances.

Accordingly, the yellow ketone of perinaphthene (benzonaphthene) was prepared according to the directions of Fieser (2) and subjected to dehydrogenation under the same conditions used for cevine (1). A hydrocarbon could be isolated readily which, however, did not give the analytical data expected for perinaphthene but rather those for a hydrocarbon with 2 more H atoms,

*viz.* perinaphthane, produced presumably by disproportionation. The melting point also agreed with that reported for perinaphthane.

Although such behavior seemed in itself to be against the likelihood of the more complex hydrocarbons discussed above withstanding the dehydrogenating effect of selenium, we have gone further and synthesized a hydrocarbon with the empirical formula of methylperinaphthene by the action of methyl magnesium iodide on perinaphthenone. Although we had expected the initial formation of an alcohol which could then be reduced and dehydrated to methylperinaphthene, a hydrocarbon among other products resulted which gave the proper analytical figures for the desired one. The mechanism of its formation is obscure but may be possibly due to the reducing action of the reagent (7). Aside from the hydrocarbon a yellow solid was also isolated which melted at 87–88° and gave analytical figures corresponding to the empirical formula of  $C_{14}H_{10}O$ . The exact nature of this product was not determined.

The hydrocarbon melted at 63–65° and proved to be somewhat unstable. Although it could be distilled in a high vacuum to give an almost colorless crystalline solid, upon standing at room temperature a green color developed in the course of a few hours. The absorption spectrum in ethyl alcohol is represented by the curve given in Fig. 4.<sup>2</sup> Comparison of this curve with the absorption spectra curves of simple naphthalene derivatives shows that in this derivative the conjugated double bond increases the absorption and also displaces the bands considerably toward the longer wave-length. This is a result in agreement with the absorption shown by propenylnaphthalene (6) and therefore cannot very well account for the type of absorption shown in Fig. 3 for the hydrocarbons from cevine.

We have further subjected the hydrocarbon methylperinaphthene to treatment with selenium under the same conditions used for the preparation of the cevine hydrocarbons in order to see whether it would withstand such treatment or possibly undergo

<sup>2</sup> In a private communication, Dr. Fieser of Harvard University has kindly sent us the absorption spectrum curve of a methylperinaphthene, possibly isomeric with our substance. This curve is in good agreement with that obtained with our substance.

a rearrangement of double bonds to the forms most stable under such conditions. The hydrocarbon isolated in rather poor yield from the resulting mixture crystallized when placed in a freezing mixture but was liquid at room temperature. The analytical data indicated formation of methylperinaphthane by addition of 2 atoms of hydrogen owing to disproportionation and were in agreement with the result of the dehydrogenation of perinaphthenone. While these results are not sufficient to rule out entirely

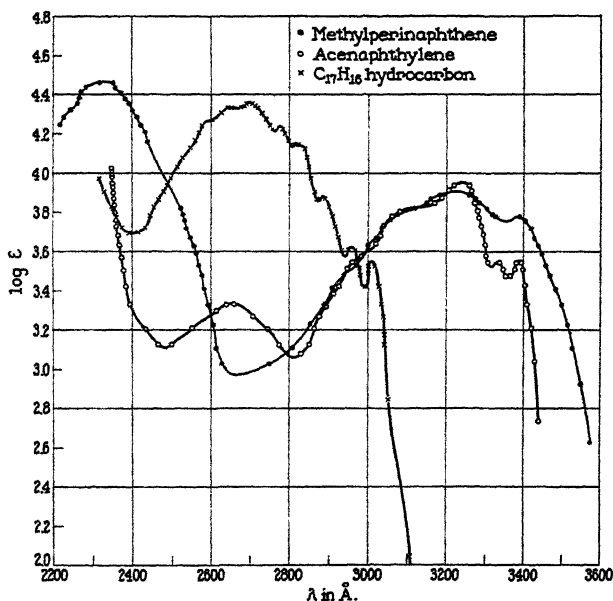


FIG. 4

such general structures as are represented in Formulas II and III, they do serve to make them seem even less likely than other general considerations would indicate.

The formulation represented in Formula IV would be that of an acenaphthylene derivative. Acenaphthene is reported not to dehydrogenate with selenium to acenaphthylene (8) but, nevertheless, the absorption spectrum of the latter was determined and is shown in Fig. 4. This curve seems to be sufficiently different from those of the hydrocarbons from cevine to remove this possibility from consideration.

Of various other known types of absorption spectra, that of diphenyl (Fig. 2) seems to offer considerable similarity to that of our hydrocarbons. The general shape of the curve is approximately the same as well as the intensity of the absorption. The curve of any one of the hydrocarbons, however, covers a region approximately 200 Å. displaced toward the longer wave-lengths from that of diphenyl. This is an effect shown by many examples in the literature to be caused by the substitution of alkyl groups or saturated rings on an aromatic nucleus. If any of the four hydrocarbons is a diphenyl derivative, two further saturated rings must be present in order to make up the ten necessary double bonds or rings and thus conform with the established empirical formulas. Thus two general possibilities remain; *viz.*, a cyclopentenofluorene derivative and the other a cyclopenteno-9,10-dihydrophenanthrene derivative. Both would be expected to give a somewhat modified diphenyl type of absorption curve.

A comparison of the curves of fluorene, 9,10-dihydrophenanthrene, and the  $C_{17}H_{16}$  hydrocarbon is shown in Fig. 5. The curves of fluorene and 9,10-dihydrophenanthrene were replotted from data taken from the curves of Askew (9).

Before a 9,10-dihydrophenanthrene derivative can be seriously considered, the possibility that such a derivative would resist dehydrogenation to the corresponding phenanthrene would have to be weighed. That such a possibility cannot be entirely dismissed may be derived from the experience of Bergmann and Weizmann (10) who found that 1,2-dimethyl-3,4,9,10,11,12-hexahydrophenanthrene as well as its 7-methoxy derivative did not dehydrogenate readily to the corresponding phenanthrene and only in poor yield upon long treatment. Oils giving the proper analytical data for dihydro derivatives were isolated which were considered by them to be 9, 10-dihydrophenanthrene derivatives. This question, however, must be explored more carefully at a future time.

Thus, the possibility must be considered that the hydrocarbons from the selenium dehydrogenation of cevine could be derivatives of a cyclopentenophenanthrene and if so, barring obscure rearrangements, that cevine could contain such a completely hydrogenated ring structure. However, the failure to dehydrogenate to a phenanthrene has not been encountered in the sterols heretofore as far as we are aware.

The other possibility, a cyclopentenofluorene derivative, would not be an unlikely product of a selenium dehydrogenation. In favor of such a structure can be mentioned the behavior of all

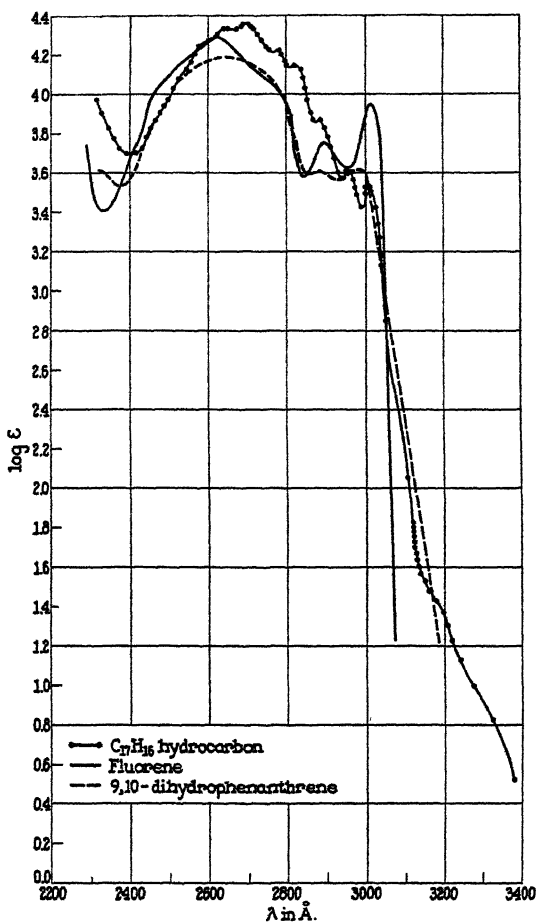


FIG. 5

four of our hydrocarbons when subjected to the Vanscheidt color test (11). When a small amount of the hydrocarbon is dissolved in pyridine, a drop of alcoholic KOH added, and the solution heated with shaking, a yellow color develops which turns gradually

green. The production of color under these conditions has been used to detect the presence of fluorenes, indenenes, and derivatives

R  
having a  $C=C-C-C=C$  grouping in hydrocarbon mixtures.  
H

As a check on the selectivity of this test, the behavior of 9,10-dihydrophenanthrene was studied and, as was to be expected, gave a negative test. The 9,10-dihydrophenanthrene was kindly placed at our disposal by Dr. Erich Mosettig.

Should these hydrocarbons prove to be fluorene derivatives and their primary character assumed, then cevine could contain a completely hydrogenated 3,4- or 1,2-cyclopentenofluorene ring system and in the latter case would be somewhat suggestive of the sterol ring system with Ring B 5-membered. However, in either case, the formation of 4,5-benzohydrindene as well as that of the naphthalic acid anhydride obtained from decevinic acid (5) by sulfur dehydrogenation could be explained only by ring enlargement of Ring 2. Both these derivatives were obtained by dehydrogenation, although under temperature conditions much lower than those usually supposed to cause ring enlargement of a cyclopenteno derivative (8). Ring 4 would be attached either as it is represented in the formula, to positions 1,2 on the fluorene nucleus or to positions 3,4 in order to permit the formation of the 4,5-benzohydrindene.

These considerations can only be of the most tentative character based on the observations which we have thus far been able to make. Modification may well become necessary as more data are obtained but our observations serve the purpose of suggesting possible further modes of approach in a field very confusing and experimentally very difficult. There is naturally a temptation to attempt to reconcile the data obtained with something approaching a sterol ring structure, since there are alkaloids already known such as the *Solanum* alkaloids which are considered to have such a structure. However, we have always been confronted with the unique and striking fact that while the hydrocarbon dehydrogenation products of cevine thus far isolated can be shown to have a relation with each other, they do not, to our knowledge, coincide with any sterol dehydrogenation product thus far isolated. Either some unique structural arrangement is responsible for such a

behavior, or, what is more probable, cevine does not bear a close relationship to the sterols.

#### EXPERIMENTAL

*Dehydrogenation of Perinaphthenone with Selenium*—1.5 gm. of perinaphthenone prepared according to the directions of Fieser and Hershberg (2) were ground with 4 gm. of selenium and heated in the customary apparatus for dehydrogenation at a temperature of  $340^{\circ}$  and in an atmosphere of nitrogen for 2 hours. The material remaining in the flask was pulverized and extracted with ether. After evaporation of the ether extract, a residue of 0.45 gm. remained. This was dissolved in 10 cc. of benzene and passed through a chromatograph prepared with 40 gm. of Brockmann's alumina in benzene. The first 100 cc. of benzene coming through the column after the solvent originally present had passed were concentrated through a Vigreux column until a syrup remained. This syrup was then placed in a small still (5 cm. column) of the type previously reported (12) and fractionated. 50 mg. of material distilled at a bath temperature of  $140^{\circ}$  and under 2 to 3 mm. pressure. This material crystallized in the condenser and melted at  $60-63^{\circ}$ . After recrystallization from ether-petroleum ether 25 mg. were obtained which melted at  $62-63^{\circ}$ . The melting point reported by Fieser and Hershberg for perinaphthane was  $65.1-65.4^{\circ}$ .

$C_{13}H_{12}$ . Calculated, C 92.80, H 7.20; found, C 92.11, H 7.04

Since the analytical data indicated impurity, the picrate was prepared and crystallized from acetone. The orange needles had a micro melting point of  $148-150^{\circ}$ . Fieser and Hershberg (2) report a melting point of  $150-151^{\circ}$ .

$C_{13}H_{15}O_7N_3$ . Calculated, C 57.43, H 3.80; found, C 57.26, H 3.51

*Addition of  $CH_3MgI$  to Perinaphthenone (Methylperinaphthene)*— $CH_3MgI$  was prepared in the customary way with 4 cc. of methyl iodide and a small excess of Mg. To this reagent was slowly added a solution of 2 gm. of perinaphthenone dissolved in a few cc. of benzene diluted with several volumes of ether. There was little evidence of reaction except that the color of the solution became reddish brown. After being refluxed for an hour the mixture

was poured on crushed ice and then acidified with HCl. The ether layer was dried over  $K_2CO_3$  and evaporated to dryness in a sublimation apparatus. 1.6 gm. of material sublimed up to a temperature of  $120^\circ$  and under 0.2 mm. pressure. It was light yellow at first but soon turned reddish brown.

The sublimate was dissolved in 10 cc. of benzene and passed through a chromatograph of 60 gm. of Brockmann's alumina in benzene. As soon as material appeared at the lower end, 30 cc. were collected and evaporated. This contained 0.8 gm. of an almost colorless oil which was set aside as Fraction 1. The next 100 cc. of eluent appeared to have a more distinct color and on evaporation yielded 500 mg. of residue which crystallized readily. On recrystallization from ether 270 mg. of stout yellow-green columns were obtained which melted at  $87-88^\circ$ . Since the analysis showed the substance to be oxygen-containing, it was not investigated further.

$C_{14}H_{10}O$ . Calculated, C 86.55, H 5.15; found, C 86.58, H 5.19

Distillation of Fraction 1 and analysis of the distillate indicated it to contain considerable amounts of an oxygen-containing substance. It was dissolved in 10 cc. of commercial isohexane and chromatographed with 60 gm. of Brockmann's alumina in isohexane. The first volume of 50 cc. containing dissolved material was collected. This yielded 300 mg. of an oil which crystallized. It distilled completely at an oil bath temperature of about  $130^\circ$  and 0.2 mm. The substance showed a micro melting point of  $60-65^\circ$  but retained a slight green color.

$C_{14}H_{12}$ . Calculated, C 93.33, H 6.67; found, C 93.46, H 6.67

If the crystalline material from the chromatograph was recrystallized directly from isohexane without distillation, the substance obtained was practically colorless and had a micro melting point of  $63-65^\circ$ . However, on standing at room temperature for several hours, color appeared and in a few days the substance changed to a dark green oil.

The absorption spectrum curve of this substance is given in Fig. 4.

*Treatment of Methylperinaphthene with Selenium*—0.5 gm. of the hydrocarbon was ground with 2 gm. of selenium and dehydro-



generated in the customary vessel in an atmosphere of nitrogen by heating for 1 hour at 340°. The pulverized reaction mixture on extraction with ether yielded an oil which was fractionated in a microdistillation apparatus. 75 mg. of colorless oil distilled up to 140° and under 1 mm. pressure. At a slightly higher temperature 50 mg. further distilled but this fraction had a slight color. The first fraction crystallized directly when chilled but melted again at room temperature. It could not be crystallized from a solvent. On standing it remained colorless. Analysis indicated

TABLE I  
*Fractionation of Acenaphthylene*

Fraction No.	Column temperature (120° bath temperature)	Weight	Micro m.p.
	°C.	mg.	°C.
1	90	50	50- 75
2	90	50	88- 91
3	90	90	89- 92
4	90	130	83- 87
5	90	140	73- 81
6	90	80	92-108
7	90	30	75- 87
8	96	140	81- 87
9	96	50	89- 93

addition of 2 atoms of hydrogen and formation of a methylperinaphthane.

$C_{14}H_{14}$ . Calculated, C 92.26, H 7.74; found, C 92.37, H 7.80

*Acenaphthylene*—This substance was prepared according to Blumenthal (13) by passing 2 gm. of acenaphthene through a hot tube filled with lead oxide. The entire distillate was crystallized in the cold from about 10 cc. of ether. 1 gm. of needles was collected which proved to be unchanged acenaphthene. The concentrated mother liquor was placed in a microfractionation apparatus (12) having a column 22 cm. in length. The fractionation was carried out under 0.2 mm. pressure (Table I). Fraction 3 was taken for analysis.

$C_{12}H_8$ . Calculated, C 94.69, H 5.31; found, C 94.64, H 5.49

Upon recrystallization from ether, golden yellow plates were obtained which had a micro melting point of 92–94°. This material was used for the absorption spectra study. The melting point reported in the literature is 92°.

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## THE VERATRINE ALKALOIDS

### X. THE STRUCTURE OF CEVANTHRIDINE

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In the previous paper (1) a discussion concerning the nature of the hydrocarbons obtained from the selenium dehydrogenation of cevine was presented on the basis of a study of their ultraviolet absorption spectra. While experiments are in progress to obtain further evidence, we have attempted to extend the same sort of study to the degradation product, cevanthridine, which appears to contain all but a few of the carbon atoms originally present in cevine.

Cevanthridine was first isolated by Blount (2) more than 5 years ago but aside from certain deductions drawn from x-ray studies of the crystals (3) in regard to the molecular dimensions nothing further has appeared which has a bearing on the structure of this substance. However, from data now available in this laboratory it is possible to obtain certain additional suggestions regarding its nature and its relationship to the simpler hydrocarbon degradation products reported by us.

The empirical formula  $C_{23}H_{25}N$  was proposed by Blount for cevanthridine on the basis of the analysis of the free base and of its methiodide. In the case of the picrate, however, the analytical data seemed to come consistently high in carbon and this substance was therefore assumed by him to contain less than the molecular ratio of picric acid. Such analytical results we have also encountered with the picrate and while our analytical results are in good agreement with those of Blount for the free base, we have consistently obtained higher carbon figures in the analysis of the methiodide. These results offer the suggestion that cevanthridine may be after all a somewhat larger molecule than originally proposed by Blount.

Our analytical figures seem to indicate rather a formulation of  $C_{25}H_{27}N$ , which is also in agreement with the analytical results reported by Blount on the picrate and the free base. This formulation is further supported by a study of the hydrogenation product resulting from the catalytic hydrogenation of cevanthridine described below, as well as of derivatives of this substance. Since

TABLE I  
*Cevanthridine and Derivatives*

Substance	Calculated						Found	
	$C_{25}H_{25}N$		$C_{24}H_{27}N$		$C_{25}H_{27}N$		C	H
	C	H	C	H	C	H		
Cevanthridine	87.56	7.99	87.48	8.26	87.92	7.97	87.70	8.10
							87.59	8.24
Methiodide	63.01	6.17	63.68	6.56	64.56	6.25	64.30	6.10
							64.19	6.39
							62.8	6.1*
Picrate	63.95	5.18	64.49	5.41	65.24	5.30	65.46	5.16
							65.65	5.31
							65.1	5.4*
							65.2	5.4*
Dihydro-	87.01	8.57	86.96	8.82	87.41	8.52	86.93	8.81
							86.84	8.89
Tetrahydro-	86.46	9.15	86.44	9.37	86.91	9.05		
Acetyldihydro-	83.52	8.14	83.60	8.37	84.11	8.11	83.84	8.39
							83.94	8.58
Acetyltetrahydro-	83.06	8.65	83.13	8.86	83.68	8.59		
Dihydro- hydrochloride	78.06	7.98	78.33	8.22	79.01	7.96	78.44	8.43
							78.57	8.33
Tetrahydro- hydrochloride	77.59	8.50	77.90	8.72	78.53	8.45		
Dihydro- <i>p</i> -bromobenzoyl-	71.98	6.04	72.36	6.27	72.99	6.13	72.66	6.87
Tetrahydro- <i>p</i> -bromobenzoyl-	71.72	6.42	72.08	6.64	72.70	6.48		

\* Results taken from the paper of Blount (2).

it is so often difficult to decide on the proper formulation of a substance of this type, Table I, containing the theoretical values for the various formulas together with the analytical results, is given.

The catalytic hydrogenation of cevanthridine has been studied as part of the investigation. When cevanthridine was hydrogenated in glacial acetic acid with the platinum oxide catalyst of

Adams and Shriner, somewhat more than 2 moles of hydrogen were readily absorbed. The hydrogenation product readily crystallized under ether and melted at 158–159°. This *tetrahydrocevanthridine* contains a nitrogen atom which must now be secondary in character, since it readily yielded an *acetyl derivative* with acetic anhydride. The acetyl derivative did not form a hydrochloride, as contrasted with the parent base which readily yielded a crystalline *hydrochloride*. The analytical data in Table I are all in conformity with a tetrahydro derivative on the basis of the  $C_{25}H_{27}N$  formula now proposed by us.

If either the formula assigned to cevanthridine by Blount or one with one  $CH_2$  more is assumed, the hydrogenated derivative could be only a dihydro derivative, since the addition of 4 hydrogen atoms to either of these formulas would result in formulations not supported by our analytical results. On the other hand, if cevanthridine should yield a dihydro derivative which possesses a secondary nitrogen atom, the only probable structure would be that of an acridine derivative in order to make such a transformation possible. It is difficult to see how an acridine derivative could be formed from an octahydropyridocoline or similar ring system (4) such as appears to be present in the cevine molecule without postulating involved degradation followed by synthesis during the selenium dehydrogenation.

A more probable explanation would seem to be that one ring of the assumed bicyclic nitrogen ring system is ruptured to give an intermediate secondary nitrogen in the ring which is joined to the remaining part of the molecule. This ring could then be dehydrogenated with the remainder of the molecule to a quinoline or isoquinoline derivative. Such a derivative would form a tetrahydro derivative under the conditions used for the hydrogenation and the formulation, therefore, of  $C_{25}H_{27}N$  for cevanthridine appears to be the most satisfactory. It is not only supported by the analytical results found with the base itself but also with those obtained with the methiodide and the picrate.

A study of the absorption spectrum of cevanthridine<sup>1</sup> has been made in order to learn if a relationship would be found to the type of absorption shown by the hydrocarbons obtained from the same

<sup>1</sup> The absorption spectra curves were very kindly determined by Dr. George I. Lavin of the Rockefeller Institute.

dehydrogenation. While an obvious relationship does not at once appear with cevanthridine itself, as comparison of Curve 1, Fig. 1, with Curve 2 of the  $C_{17}H_{16}$  hydrocarbon will show, the absorption spectrum of the tetrahydro derivative, Curve 3, is strikingly similar to that of the hydrocarbon. The conclusion may therefore be drawn that the same ring structure present in the hydrocarbon is probably also present in the tetrahydro derivative of cevanthridine. Further, since the substitution of a nitrogen directly on an aromatic ring can greatly change the absorption spectrum

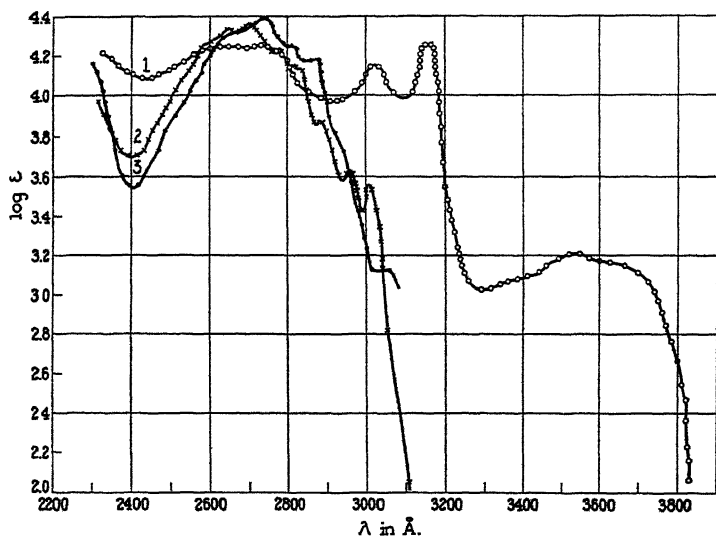


FIG. 1. Absorption spectra of cevanthridine (Curve 1),  $C_{17}H_{16}$  hydrocarbon (Curve 2), and tetrahydrocevanthridine (Curve 3).

as demonstrated for example by the difference in the absorptions shown by naphthalene and aminonaphthalene (5), the nitrogen in tetrahydrocevanthridine may be inferred to be at least 1 carbon atom removed from the aromatic nucleus, such as is found in a tetrahydroisoquinoline derivative.

In the previous paper (1), of the three structures discussed as more or less in harmony with the absorption spectra and properties of the hydrocarbons isolated from the dehydrogenation of cevine, a cyclopentenofluorene structure appears perhaps most satisfactory. On such a basis, it is possible that cevanthridine may

have a structure in which an additional 6-membered nitrogen ring is joined at some position on either of the benzene rings and thus may form an isoquinoline derivative. Since alkaloids related to the sterols are known to occur, it might be considered possible that the position of the nitrogen ring could be as shown in Formula I.

But in the attempt to reconcile such possibilities with Ring 2, whether 5- or 6-membered, it is still difficult to see how an octahydropyridocoline or such bicyclic saturated tertiary base could break away intact from two other rings such as Ring 3 or 4 on pyrolysis. It may be that the union between the bicyclic basic portion of the alkaloid and the remainder of the molecule is by means of a single carbon-to-carbon bond and that the formation of the nitrogen ring of cevanthridine on pyrolysis is due to secondary ring closure. In any event much more work will have to be done to correlate properly all of the data at hand.

The empirical formula of cevine is  $C_{27}H_{45}O_8N$ . This formula when related back to the saturated hydrocarbon without rings implies a total of seven rings plus double bonds. Although originally the possibility had been considered that it might contain a lactone group because of the ability to form a potassium derivative (6), later study has failed to give any evidence of the presence of the necessary carboxyl group. On the other hand, there is a possibility that the presence of an enolic hydroxyl group (7) could account for the formation of a potassium derivative. Catalytic hydrogenation studies (7) have revealed the presence of a single unsaturated linkage and perhaps the one responsible for the enolic hydroxyl (ketone?) group. Absorption spectra studies have failed to reveal absorption bands in the region where benzenoid substances are known to absorb. It would appear, therefore, that there are no double bonds present aside from the one mentioned. In general conformity with this assumption is the failure to isolate anything benzenoid from among the products of the soda lime or zinc dust distillation as well as the number of rings found to be present in the hydrocarbons from the selenium dehydrogenation. Accordingly the ring structure of cevine can be assumed to be hexacyclic.

Four of these six rings may be accounted for by two of the degradation products; namely, the so called octahydropyridocoline fragment and decevinic acid. Together these two derivatives



account for 24 of the 27 carbon atoms originally present in cevine and may be joined to each other in such manner that two further rings are formed. At least one of these rings is very likely 5-membered and appears in the benzohydrindene hydrocarbon. On the basis of the structures postulated for the other hydrocarbons from the selenium dehydrogenation the remaining ring must be 6-membered. Thus all the rings could be accounted for and there would remain the manner in which they are joined to be determined.

#### EXPERIMENTAL

*Cevanthridine Methiodide*—30 mg. of cevanthridine when treated with excess methyl iodide at first dissolved but soon deposited crystals. After the mixture was refluxed for several hours, the crystalline material was collected. The melting point was 268–270°, depending somewhat on the rate of heating, and was not changed by recrystallization from acetone. Blount (2) reported 255–256°.

$C_{26}H_{30}NI$ . Calculated, C 64.56, H 6.25; found, C 64.30, H 6.10  
“ 64.19, “ 6.39

*Hydrogenation of Cevanthridine*—60 mg. of cevanthridine were hydrogenated in 3 cc. of glacial acetic acid with 50 mg. of platinum oxide catalyst. After the mixture was shaken under approximately 3 atmospheres of hydrogen for 1.3 hours, 2 moles of hydrogen had been absorbed and the absorption became slow. It was interrupted at this point although previous experiments had shown that hydrogenation gradually proceeded further. The filtrate from the catalyst was evaporated to dryness under reduced pressure. After treatment with NaOH solution, the base was extracted with ether. This yielded on concentration 40 mg. of crystalline material which melted at 151–156°. Upon repeated recrystallization a melting point of 158–159° was obtained.

$C_{26}H_{31}N$ . Calculated, C 86.91, H 9.05; found, C 86.93, H 8.81  
“ 86.84, “ 8.89

When a suspension of cevanthridine in a few cc. of ethyl alcohol containing a slight excess of HCl was hydrogenated, the hydrochloride was directly obtained on concentration. 25 mg. of well

formed crystalline blades were collected. The material did not possess a well defined melting point but sintered and decomposed from 280–295°, depending somewhat on the rate of heating. Neither the analytical data nor the melting point changed upon recrystallization.

$C_{26}H_{31}N \cdot HCl$ . Calculated, C 78.53, H 8.45; found, C 78.57, H 8.33  
" 78.44, " 8.43

*Acetyltetrahydrocevanthridine*—20 mg. of the tetrahydro derivative were refluxed for 2 hours in acetic anhydride. After concentration the residue was dissolved in ether. The solution was extracted with dilute HCl and dried. On concentration of the ether solution crystalline material appeared. It melted at 202–203° and after repeated recrystallization at 206–207°.

$C_{27}H_{33}ON$ . Calculated, C 83.68, H 8.59; found, C 83.84, H 8.39  
" 83.94, " 8.58

*p*-Bromobenzoyltetrahydrocevanthridine—Approximately 30 mg. of the tetrahydro derivative were treated with 30 mg. of *p*-bromobenzoyl chloride and 2 cc. of 10 per cent NaOH. After the mixture was warmed somewhat to destroy the excess chloride, the product was extracted with ether. The substance separated from ether-petroleum ether and showed a micro melting point of 107–113°.

$C_{32}H_{34}ONBr$ . Calculated, C 72.70, H 6.48; found, C 72.66, H 6.87

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## THE PHOTOCHEMICAL SPECTRUM OF THE PASTEUR ENZYME IN RETINA\*

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The aerobic glycolysis of mammalian tissues is increased by carbon monoxide. This effect is reversible and photosensitive (2, 3). A primary CO inhibition of respiration is not the cause, since the respiratory rate remains unaltered under these conditions (3). The hypothesis that the Pasteur reaction, *i.e.* the check of fermentation by oxygen, is mediated by a specific catalyst containing heavy metal is strengthened by the selective inhibition of the effect by ethyl isocyanide (4). The term *Pasteur enzyme* is herewith proposed for this thermolabile (5), negative catalyst. In order to determine the chemical constitution of the enzyme, its spectrum has been studied by Warburg's photochemical method (6). This method utilizes the affinity of ferrous iron for carbon monoxide and the reversible photodissociation of iron-carbonyl complexes. Since only that fraction of incident light which is absorbed can exert a chemical effect, the efficiency of monochromatic radiation of constant intensity will be proportional to the degree of absorption of light of a given wave-length by the FeCO complex. When continuous illumination is used and the photochemical effects of the various wave-lengths are referred to one wave-length chosen arbitrarily as the reference standard, the *relative* spectrum of the catalyst is obtained. This yields the

\* This work was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research. A preliminary report has been published (1). Some of the data were presented before the Thirty-fourth annual meeting of the American Society of Biological Chemists (1940).

† Finney-Howell Research Foundation Fellow, 1939-41.

pattern and the positions of the absorption maxima. The *absolute* spectrum in terms of extinction coefficients referred to 1 gm. atom of iron is determined with the aid of intermittent illumination. The photochemical efficiency spectrum has been shown to be identical with the absorption spectrum of iron-carbonyl complexes (7). The present paper deals with the relative spectrum of the Pasteur enzyme in the region from 405 to 655 m $\mu$ .

The arrangement of the experiments is briefly the following. Rat retinas are suspended in a medium containing bicarbonate and glucose; they are then equilibrated in the dark with a gas mixture containing CO, O<sub>2</sub>, and CO<sub>2</sub>. With the inhibition of the Pasteur effect by CO, the already considerable aerobic glycolysis of this tissue is further increased to values approaching the level of anaerobic glycolysis. Each molecule of lactic acid formed by the tissue liberates 1 molecule of CO<sub>2</sub> from the bicarbonate of the medium. The resulting increase in pressure is measured with the aid of a differential manometer. Upon illumination with monochromatic light of known, high intensity, the rate of lactic acid production and of the subsequent CO<sub>2</sub> formation is decreased to an extent depending on the intensity and the wave-length of the radiation employed. The photochemical efficiency of each wave-length is compared with the effect of the reference wave-length 436 m $\mu$  on the same retinas.

#### EXPERIMENTAL

##### *Influence of CO on Metabolism of Retina Tissue*

Laser's experiments on the effect of CO on tissue metabolism (3) were conducted at 38°; Warburg and Negelein (8), however, had already demonstrated a marked effect of light on the glycolysis of CO-treated rat retinas at 25°. In view of the finding of Kubowitz and Haas (9) that the light sensitivity of the CO compound of the respiratory enzyme is increased upon lowering the temperature, the present photochemical experiments were carried out at 26.6°. This made it necessary to repeat the experiments of Laser at this temperature. With Warburg's two vessel method and Summerson's modification of the Dixon-Keilin method, it was found that at 26.6°, just as at 38°, the respiration is not affected by replacing a gas mixture of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub> by one of 10 per cent O<sub>2</sub>, 5 per cent CO<sub>2</sub>, and 85 per cent CO.

However, the glycolysis approaches the anaerobic level in the presence of the CO. In the absence of O<sub>2</sub>, CO has no stimulating effect on glycolysis. The data are summarized in Table I.

### *Photochemical Apparatus and Procedure*

The arrangement of the photochemical apparatus is schematically represented in Fig. 1. It follows essentially the design of that used by Warburg and his associates (9, 11).

The glycolysis is measured with the aid of a differential manometer with cylindrical vessels of the form sketched. Four to

TABLE I  
*Selective Inhibition of Pasteur Reaction in Retina by CO*

Atmosphere	At 26.6° (present experiments)		At 33° (Laser (3, 10))
	Two vessel method of Warburg	Dixon-Keilin- Summerson method	
	Q <sub>G</sub>	Q <sub>G</sub>	Q <sub>G</sub>
95% N <sub>2</sub> , 5% CO <sub>2</sub> .....	24*	30*	88
95% CO, 5% " .....	25*		
85% " 10% O <sub>2</sub> , 5% CO <sub>2</sub> .....	22	31.5	79
85% N <sub>2</sub> , 10% " 5% " .....	16	18	70
95% O <sub>2</sub> , 5% CO <sub>2</sub> .....	11	12	45
	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>	Q <sub>O<sub>2</sub></sub>
85% CO, 10% O <sub>2</sub> , 5% CO <sub>2</sub> .....	9	9	31
85% N <sub>2</sub> , 10% " 5% " .....	9	11	31
95% O <sub>2</sub> , 5% CO <sub>2</sub> .....	10	9.5	31

\* These values were obtained with simple Warburg-Barcroft manometers at the same time when the other determinations in the same series were performed.

six freshly removed rat retinas are placed in one vessel containing 2 cc. of glucose-Ringer-bicarbonate solution. The Ringer's solution contains 96 cc. of 0.9 per cent NaCl, 2 cc. of 1.22 per cent CaCl<sub>2</sub>, 2 cc. of 1.15 per cent KCl, 20 cc. of 1.3 per cent NaHCO<sub>3</sub> (through which CO<sub>2</sub> has been previously passed), and 240 mg. of glucose. A control experiment in which the glucose concentration was increased 3-fold (600 mg. per 100 cc. of Ringer's solution) yielded the same results as with the smaller glucose concentration, which indicates that during the photochemical experiments the enzyme systems concerned were saturated with substrate. The

first experimental reading is taken 20 to 25 minutes after the gas flow has been stopped, and the pressure has been equilibrated against the atmosphere. The compensation vessel contains 2 cc. of the Ringer's solution. The volume of each vessel is 20.2 cc., so that the ratio of gas volume to fluid volume is large. Under these conditions the respiratory exchange produces no appreciable pressure changes; the positive pressures recorded result, therefore,

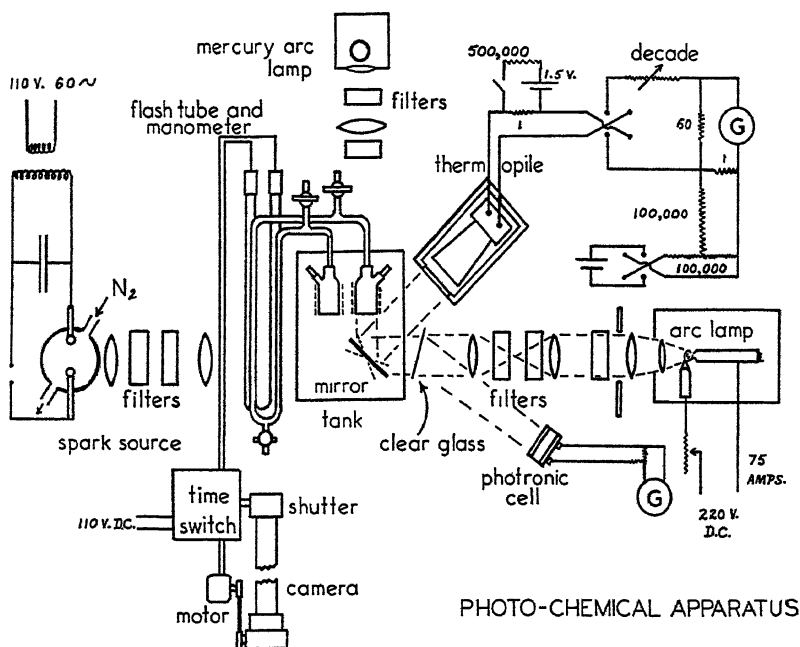


FIG. 1. Schematic diagram of the photochemical apparatus (not drawn to scale).

from lactic acid formation and the subsequent liberation of carbon dioxide from the bicarbonate-containing medium.

The cross-section of the manometer capillary is 0.5 sq. mm.; the capillary is filled with isocaproic acid (11,160 mm. = 760 mm. of Hg) or with Brodie's fluid (10,000 mm. = 760 mm. of Hg). To facilitate the filling of the capillary a stop-cock is inserted at the base of the U-shaped capillary tube.

The manometer is rotated around its vertical axis by means of

a motor in connection with an eccentric disk, with the result that the vessels make excursions of 1 to 2 mm. amplitude about a fixed point in the thermostatically controlled bath. The usual rate of rotation is 350 revolutions per minute. The pressure changes taking place are automatically recorded during the experiment by photographing the levels of the manometer fluid at regular intervals with a camera fitted with a specially corrected long focus lens of 5 cm. aperture ( $f = 82$  cm.). The magnification ratio is 0.8. The photographic recording process is controlled by a clockwork in conjunction with relays and a solenoid switch. When the minute hand of the clock makes contact with metal posts which can be spaced at distances corresponding to intervals of 1 to 10 minutes, an electromagnet becomes energized, and the following sequence of events takes place. (1) The shutter of the camera opens. (2) A gas-filled fluorescent sign tube mounted on the back of the manometer is lighted for a fraction of a second by means of a 110 volt d.c. impulse sent through a transformer (9000 volts; 0.045 ampere). (3) The shutter is closed. (4) The breaking of the direct current connection through the transformer causes the sign tube to flash again. (5) The film in the camera is advanced by a motor to receive the next image. (6) The motor is stopped automatically. The flash of the sign tube caused by the "make" charge during step (2) is rapid enough and of sufficient intensity to record an unblurred image of the levels of the manometer fluid on the film (Eastman positive, 35 mm.) without stopping the motion of the manometer. The second flash (step (4)) is not recorded because the shutter has been closed in the meantime. The films are later read either with the aid of a Bausch and Lomb measuring magnifier or by projection of the film on a graduated screen.

#### *Isolation of Monochromatic Radiation*

The light sources used include a high pressure mercury arc lamp (General Electric, Type H-3), an electrical sodium lamp (General Electric Vapor Lamp Company, sodium Labare), and a high intensity carbon arc lamp modeled after that of Kubowitz and Haas (9). The arc is operated on 220 volts d.c. and 50 to 75 amperes. It is stabilized by an air draft produced by an oil burner fan, a pair of electromagnets on either side of the arc, and by



mechanically rotating the water-cooled anode during the experiment. The light emission is varied by means of anode carbons, the cores of which are filled with various salts (Ca, Sr, Li, Mg, Cu).<sup>1</sup> The cathode carbons were usually copper-coated. A diagram of the lamp will be found in the paper of Kubowitz and Haas (9). During the experimental light periods the carbon arc is regulated by advancing the cathode and the rotating anode by hand. By reflecting, with an inclined clear glass plate inserted in the light path, a small part of the light upon a Weston photonic cell connected to a galvanometer, it is possible to keep the light intensity constant by maintaining a constant deflection of this galvanometer through manual control of the electrodes. This is facilitated by projecting an enlarged image of the burning electrodes on a screen. With the aid of Dr. L. H. Ott, an attempt was also made to utilize high voltage sparks between magnesium and tungsten electrodes in nitrogen and carbon dioxide atmospheres respectively. However, the equipment available to us yielded radiation of insufficient intensity. The light coming from the various sources is rendered parallel by suitable biconvex glass lenses made by Bausch and Lomb. The light filters employed include polished, colored glasses (Corning and Schott filter glasses), and solutions of dyestuffs<sup>2</sup> and inorganic salts contained in plane-parallel cemented glass cells. The mirrors are first surface silver mirrors on optical flats (Bausch and Lomb). All optical parts are mounted on riders on optical benches (Fuess). The manometer vessels are immersed in a closely controlled thermostat equipped with glass windows. The light enters the vessel containing the tissue from below with the aid of an adjustable mirror placed in the tank. The illumination is uniformly spread over an area larger than that covered by the vessel during its excursions. The intensity of the light in gm. calories per sq. cm. per minute is measured before and after each experiment by means of a carefully shielded, calibrated Moll thermopile (Kipp and Zonen) in conjunction with a Leeds and Northrup mirror galvanometer (sensitivity, 0.1 microvolt = 10 mm. deflection). By connecting a 1.5 volt battery in series with a 500,000 ohm resistance, and then placing this circuit in parallel

<sup>1</sup> The special carbon electrodes used in this work were made by the Siemens-Plania works which had furnished the carbons for Warburg's work.

<sup>2</sup> A number of the dyes used were kindly given to us by the National Aniline and Chemical Company, Inc.

with a 1 ohm resistance in the thermopile-galvanometer circuit, one may check the sensitivity of the galvanometer during the energy measurements. Because of zero drifts of the galvanometer it is necessary to admit an opposing small voltage (of the order of  $10^{-6}$  volt) which is controlled by a potentiometer as illustrated in Fig. 1.

The intensity of the light beam is measured before it enters the thermostat. The loss due to reflection and absorption by the windows and water is estimated with the aid of a photoelectric cell (Weston) which may be held in the bath in place of the experimental vessel. A record of the spectral purity of the radiation is obtained with a Hilger glass spectrograph. The photometry of the spectrograms enables one to determine the spectral purity of the light. The wave-lengths are checked by photographing the mercury spectrum in juxtaposition with the spectrum of the experimental radiation. The absence of infra-red light is ascertained by photographing the spectrum of the filtered radiation on infra-red-sensitive plates (Eastman No. III-L). Table II lists the wave-lengths of light employed in the present study, together with the source of light, the filters necessary for their isolation, and the intensity of the light beam before entering the thermostat.

#### *Determination of Photochemical Efficiency Ratios ( $\beta_{\lambda}/\beta_{436}$ )*

The method of charting photochemical absorption spectra has been described by Warburg (6). In the present case the photochemical effect consists in the relief of the CO inhibition of the Pasteur reaction; that is to say, glycolysis in the presence of 85 per cent CO and 10 per cent O<sub>2</sub>, in the dark, is enhanced, and the illumination of the system tends to diminish the rate of glycolysis.

When the intensities of two wave-lengths of light which will produce the same photochemical effect are known, the *relative* light absorption coefficient as referred to a standard wave-length may be calculated, for the ratio of the absorption coefficients is the reciprocal of the ratio of the light intensities times the wave-lengths.

$$\beta_1/\beta_2 = i_2\lambda_2/i_1\lambda_1$$

This equation is based on fundamental quantum relationships and has been experimentally verified for the case of FeCO complexes by Warburg and Negelein (7).

An additional factor must be considered in the case of retina,

TABLE II

*Isolation of Monochromatic Light Used in Photochemical Experiments*

Wave-length	Light source	Filters	Intensity $\times 10^4$
$m\mu$			<i>gm. calorie per sq. cm. per min.</i>
405	Mercury lamp	Noviol shade O, 2.7 mm., Corning No. 306; red-purple ultra, 3.8 mm., Corning No. 597; light blue-green, 2.0 mm., Corning No. 428	4.2
407	Strontium-carbon	Water, 5 cm.; ammoniacal copper sulfate (25 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ + 1000 cc. $\text{H}_2\text{O}$ + 55 cc. 25% $\text{NH}_4\text{OH}$ ), 2.5 cm.; noviol shade O, 2.7 mm., Corning No. 306; red-purple ultra, 3.8 mm., Corning No. 597	3.0
436	Mercury lamp	Extra light aklo, 2.3 mm., Corning No. 395; noviol shade A, 2.5 mm., Corning No. 038; violet, 2.0 mm., Corning No. 511; ammoniacal copper sulfate (as 407 $m\mu$ )	1.5-4.0
428-459*	Calcium-carbon	Water, 5 cm.; copper sulfate,† 10%, 2.5 cm.; extra light aklo, 2.3 mm., Corning No. 395; violet, 2.0 mm., Corning No. 511; noviol shade A, 2.5 mm., Corning No. 038	1.5
430-470‡	Copper-carbon	Water, 5 cm.; ammoniacal copper sulfate (as 407 $m\mu$ ); copper sulfate, 10%, 2.5 cm.; noviol shade A, 2.5 mm., Corning No. 038; violet, 2.0 mm., Corning No. 511; dark blue-green, 3.9 mm., Corning No. 430	1.1
431-462§	Strontium-carbon	Same as 430-470 $m\mu$ from copper-carbon	1.0
457	Magnesium-carbon	Water, 5 cm.; ammoniacal copper sulfate (as 407 $m\mu$ ); noviol shade A, 2.5 mm., Corning No. 038; dark blue-green, 3.9 mm., Corning No. 430; blue-purple ultra, 3.1 mm., Corning No. 585; extra light aklo, 2.3 mm., Corning No. 395	2.3

TABLE II—Continued

Wave-length	Light source	Filters	Intensity $\times 10^4$
<i>mμ</i>			<i>gm. calorie per sq. cm. per min.</i>
460	Lithium-carbon	Water, 5 cm.; ammoniacal copper sulfate (as 407 <i>mμ</i> ); noviol shade A, 2.5 mm., Corning No. 038; extra light aklo, 2.3 mm., Corning No. 395	5.8
487, 497¶	Strontium-carbon	Water, 5 cm.; Guinea green B** (18 mg. in 100 cc.), 2.5 cm.; extra light aklo, 2.3 mm., Corning No. 395; H. R. lantern blue, 2.8 mm., Corning No. 554; noviol shade C, 4.5 mm., Corning No. 338	4.3
494	Magnesium-carbon	Water, 5 cm.; Guinea green (as 487 <i>mμ</i> ); light blue-green, 2.0 mm., Corning No. 428; H. R. lantern blue, 2.8 mm., Corning No. 554; noviol shade C, 4.5 mm., Corning No. 338	3.8
517	" "	Water, 5 cm.; copper sulfate, 10%, 2.5 cm.; Guinea green (as 487 <i>mμ</i> ); noviol shade D, 2.0 mm., Corning No. 338D	4.6
515, 522††	Copper-carbon	Same as 517 <i>mμ</i> + extra light aklo, 2.3 mm., Corning No. 395	7.5
525	Strontium-carbon	Water, 5 cm.; Guinea green (as 487 <i>mμ</i> ); tartrazine** (64 mg. + 100 cc. water), 2.5 cm.; light blue-green, 2.0 mm., Corning No. 428	10.9
546	Mercury lamp	Extra light aklo, 2.3 mm., Corning No. 395; didymium, 5.0 mm., Corning No. 512; H. R. illusion pink, 4.6 mm., Corning No. 592; yellow shade yellow, 2.0 mm., Corning No. 351; copper sulfate, 10%, 2.5 cm.	13.6
553	Magnesium-carbon	Water, 5 cm.; copper sulfate, 24%, 2.5 cm.; H. R. yellow shade yellow, 2.0 mm., Corning No. 351; didymium, 14.0 mm., Jena No. BG-11	5.1

TABLE II—*Continued*

Wave-length	Light source	Filters	Intensity $\times 10^4$
<i>m<math>\mu</math></i>			<i>gm. calorie per sq. cm. per min.</i>
560	Calcium-carbon	Water, 5 cm.; copper sulfate, 10%, 2.5 cm.; H. R. yellow shade yellow, 2.0 mm., Corning No. 351; didymium, 14.0 mm., Jena No. BG-11; green, 2.0 mm., Jena No. VG-3	7.1
578	Mercury lamp	Extra light aklo, 2.3 mm., Corning No. 395; H. R. red shade yellow, 2.0 mm., Corning No. 348; copper sulfate, 10%, 2.5 cm.	3.6
582	Strontium-carbon	Water, 5 cm.; copper sulfate, 24%, 2.5 cm.; H. R. red shade yellow, 2.0 mm., Corning No. 348	2.8
589	Sodium lamp	Ferrous sulfate, †† 20% in 10% H <sub>2</sub> SO <sub>4</sub> , 2.5 cm.; H. R. red shade yellow, 2.0 mm., Corning No. 348	5.4
597	Strontium-carbon	Water, 5 cm.; copper sulfate, 10%, 2.5 cm.; H. R. lighthouse red, 3.2 mm., Corning No. 246	7.1
640-655	" "	Water, 5 cm.; ferrous sulfate (as 589 m $\mu$ ); copper sulfate, 10%, 2.5 cm.; H. R. signal red, 2.1 mm., Corning No. 243	7.6
640-650	Calcium-carbon	Same as 640-655 m $\mu$ from strontium-carbon	9.5

The thickness of the impregnated anode carbons was 20 mm. except in the instance of the lithium-carbon which was 12 mm. The cathode carbons were copper-coated and, for the most part, copper-filled. Their thickness varied from 8 mm. to 14 mm., 8 mm. being the size usually employed. These latter electrodes were kindly supplied by the National Carbon Company, Inc., Cleveland, Ohio.

\* The following lines are included: 428, 429, 430, 431, 432, 436, 443, 453, 458, and 459 m $\mu$ .

† CuSO<sub>4</sub>·5H<sub>2</sub>O.

‡ The multiple line spectrum and the strong background emission in this region produce a fairly continuous spectrum between these wave-lengths.

§ Lines at 431, 433, 434, 436, 444, 453, 457, and 462 m $\mu$ , and rather pronounced background emission.

TABLE II—*Concluded*

|| Contains about 30 per cent impurities consisting of 460, 455, 452, 451, 448, 443, 441, and 436  $m\mu$ .

¶ Only these two lines present; very little background emission.

\*\* Obtained from the National Aniline and Chemical Company, Inc., Guinea green B, No. 433, tartrazine, No. 94.

†† Small impurity at 529  $m\mu$ .

‡‡  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ .

for the metabolic rate of this tissue falls with time independently of CO or illumination. Therefore it is necessary to determine the "dark glycolysis" of the retinas before and after each light period. By graphical interpolation it is then possible to determine what the glycolysis of the retina would have been, if the light had not been allowed to enter the system. The value of the "dark glycolysis" thus obtained is used to calculate the photochemical efficiency ( $A$ ) of a particular wave-length of known intensity.

$$A = \frac{(\Delta p / \Delta t)_d - (\Delta p / \Delta t)_l}{(\Delta p / \Delta t)_d}$$

where  $A$  = photoactivity;  $(\Delta p / \Delta t)_d$  = change in pressure due to the glycolysis in the dark for the period  $\Delta t$ ; and  $(\Delta p / \Delta t)_l$  = change in pressure due to the glycolysis in the light for  $\Delta t$ .

The same intensity of the same wave-length of light does not invariably produce an identical photochemical effect in two different experiments. This difficulty is overcome by determining the light sensitivity of the retinas in each experiment. This is done by illuminating, during each experiment, with *two* different intensities of blue light at 436  $m\mu$ . From the light activities which one determines for these two light intensities, one may plot intensity at 436  $m\mu$  against the photoactivity at 436  $m\mu$ . During the experiment between the two periods of illumination with 436  $m\mu$ , the retinas are subjected to the action of the "unknown" wave-length; unknown in the sense that all the absorption coefficients are referred to the coefficient at 436  $m\mu$  which is arbitrarily fixed at 1.0. From the calibration curve of the experiment and from the photoactivity of a given intensity at the unknown wave-length, the intensity at 436  $m\mu$  which would produce the same photoactivity is found. Over 100 photochemical experiments have been performed; the protocol of a typical experiment is presented in Table III.

TABLE III

*Photochemical Effect of Radiation at Wave-Length 436 m $\mu$  Versus 546 m $\mu$  on  
CO Inhibition of Pasteur Reaction in Rat Retina*

Protocol of Experiment 74 (March 16, 1940).

Time	Light		Manometer deflections $\Delta p/3$ min.
	Wave-length	Intensity	
<i>min.</i>	<i>m<math>\mu</math></i>	<i>gm. calorie per sq. cm. per min.</i>	<i>mm.</i>
0	546	$8.6 \times 10^{-4}$	4.03
3			
6			4.15
10			2.93
13			
16			
20	436	$1.3 \times 10^{-4}$	3.77
23			
26			3.38
30			2.57
33			
36			
42	546	$8.6 \times 10^{-4}$	3.12
45			
48			3.12
52			2.22
55			
58			
62	436	$3.7 \times 10^{-4}$	2.60
65			
68			2.73
72			1.82
75			
78			
82	436	$3.7 \times 10^{-4}$	2.47
85			
88			

*Evaluation of Experiment 74 (Table III)*—From Fig. 2 in which the glycolysis in the dark is plotted against time, there are obtained the values for  $(\Delta p/\Delta t)_d$  for the time when  $(\Delta p/\Delta t)_l$  was being measured (Table IV, Column 3). From these values and the

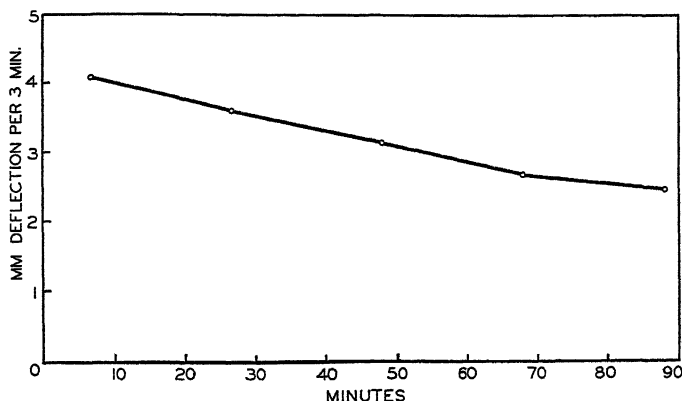


FIG. 2. Rate of glycolysis in the dark

TABLE IV

*Photoactivity, As Calculated from Data Given in Table III, 436 mμ Versus 546 mμ*

Time	Light		$(\Delta p/3 \text{ min.})_d$	$(\Delta p/3 \text{ min.})_l$	$\frac{A}{\frac{(\Delta p/3 \text{ min.})_d}{(\Delta p/3 \text{ min.})_l} \times 100}$
(1)	(2)		(3)	(4)	(5)
min.	mμ	gm. calories per sq. cm. per min.	mm.	mm.	per cent
16	546	$8.6 \times 10^{-4}$	3.82	2.93	23.2
36	436	$1.3 \times 10^{-4}$	3.32	2.57	22.6
58	546	$8.6 \times 10^{-4}$	2.88	2.22	22.8
78	436	$3.7 \times 10^{-4}$	2.57	1.82	29.2

experimentally determined  $(\Delta p/\Delta t)_l$  values the photoactivity ratios,  $A$ , were calculated as indicated in Table IV.

In Fig. 3 the photoactivity at 436 mμ is plotted against the intensity at 436 mμ. From this calibration curve, it is found that a photoactivity of 23.0 per cent (average of the two 546 mμ values which were obtained with the same intensity at 546 mμ) would be



produced by  $1.34 \times 10^{-4}$  gm. calorie per sq.cm. per minute at 436  $m\mu$ . This means that an intensity of  $1.34 \times 10^{-4}$  at 436  $m\mu$  is equivalent to an intensity of  $8.6 \times 10^{-4}$  at 546  $m\mu$ . The relative absorption coefficient at 546  $m\mu$ , then, is

$$\frac{\beta_{546}}{\beta_{436}} = \frac{1.34 \times 10^{-4} \times 436}{8.6 \times 10^{-4} \times 546} = 0.124$$

Four experiments at  $\lambda = 546 m\mu$  yielded a mean value of 0.11 for  $\beta_{546}/\beta_{436}$ ; Warburg and Negelein in their measurements on retina found this ratio to be 0.116.

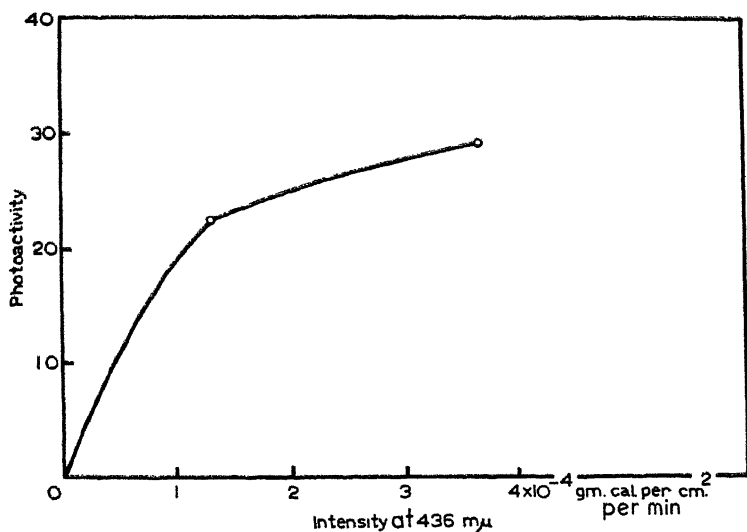


FIG. 3. Photoactivity curve at 436  $m\mu$

### Results

The relative absorption coefficients, as determined in the manner just described for twenty-one different wave-lengths in the visible region from 405 to 655  $m\mu$ , are presented in Table V. From these data, the relative absorption spectrum of the Pasteur enzyme-CO compound may be constructed (see Fig. 4).

In two instances composite  $\beta$  values were determined for a pair of lines. For the strontium lines at 487 and 497  $m\mu$ , a  $\beta$  value of 0.24 was found; the contour of the absorption curve charted without these two points indicates individual  $\beta$  values of

TABLE V  
Relative Absorption Coefficients of Pasteur Enzyme

Wave-length	Light source	$\beta_{\lambda}/\beta_{436}$	Wave-length	Light source	$\beta_{\lambda}/\beta_{436}$
$m\mu$			$m\mu$		
405	Hg	0.39	497	Sr	0.27
407	Sr	0.42	515	Cu	0.42
436	Hg	1.00	517	Mg	0.34
428-459	Ca	1.85	522	Cu	0.20
430-470	Cu	2.02	525	Sr	0.08
431-462	Sr	1.90	546	Hg	0.11
450	Cu	4.0*	553	Mg	0.15
450	Sr	3.75*	560	Ca	0.14
457	Mg	1.35	578	Hg	0.75
460	Li	1.15	582	Sr	0.46
487	Sr	0.20	589	Na	0.26
494	Mg	0.09	597	Sr	0.15
			640-655	"	0.00
			640-650	Ca	0.00

\* Calculated; see the text.

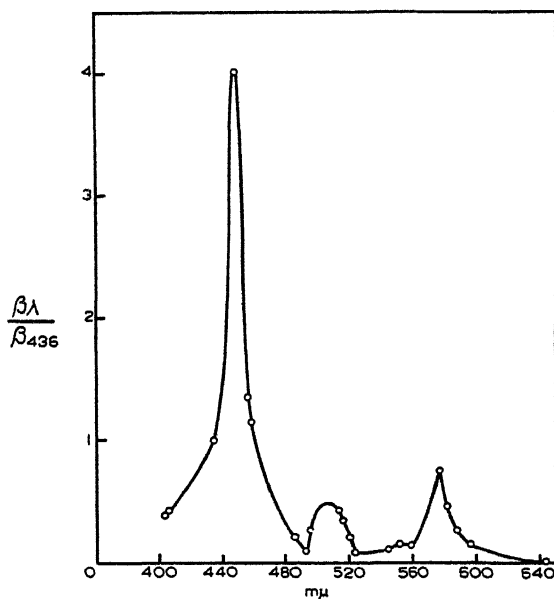


FIG. 4. Relative photochemical absorption spectrum of the CO compound of the Pasteur enzyme in rat retina.

0.20 and 0.27 for 487 and 497  $m\mu$  respectively. Similarly, the value for the effect of the combined 515 and 522  $m\mu$  lines ( $\beta = 0.31$ ) was found to equal the average of the two values for these lines as deduced from the absorption curve ( $\beta_{515} = 0.42$ ;  $\beta_{522} = 0.20$ ).

The determination of the  $\beta_{450}/\beta_{436}$  ratio presented a more difficult problem. It was not possible to isolate any suitable single wave-length in the region between 436 and 457  $m\mu$ . Although the 448  $m\mu$  line was isolated from a high tension Mg spark in a nitrogen atmosphere (11), the intensity was not sufficient. However, with the carbon arc lamp and Cu-, Sr-, and Ca-impregnated carbons a band of radiation was obtained covering this region; in all three cases the composite  $\beta$  value was about twice that at 436  $m\mu$ . This indicated that the maximum of the main absorption band of the catalyst is contained in this region of the spectrum and that the  $\beta$  value is more than twice that of 436  $m\mu$ . The contour of the band suggested that the maximum is located in the neighborhood of 450  $m\mu$ ; the relative height of the band was estimated in the following manner.

The spectrogram of the 425 to 480  $m\mu$  region of the Cu-carbon emission as obtained by filtering out the rest of the copper spectrum was examined with a microphotometer, and the intensity distribution curve was obtained (Fig. 5). For the maximum of the main band various values were assumed, and the composite  $\beta$  value was computed for the region 425 to 480  $m\mu$ , with due attention paid to the contribution of the energy furnished by each wave-length in the filtered copper radiation. From Table VI it will be seen that when the relative absorption coefficient of the 450  $m\mu$  band is set at 4, then a theoretical  $\beta_{425-480}/\beta_{436}$  value of 2.07 is obtained. The average of four experimental determinations was 2.02.

When the data obtained for the Sr spectrum in this region were treated in similar fashion, a  $\beta_{450}/\beta_{436}$  ratio of 3.75 was found. The result of the procedure employed here depends on the *shape* of the absorption band. In computing the above values it has been assumed that the  $\gamma$ -band of the Pasteur enzyme has the same shape as that of other iron porphyrin-proteins; *e.g.*, hemoglobin and the respiratory ferment of yeast. This assumption appears reasonable in view of the shape of the lower portion of the  $\gamma$ -band as

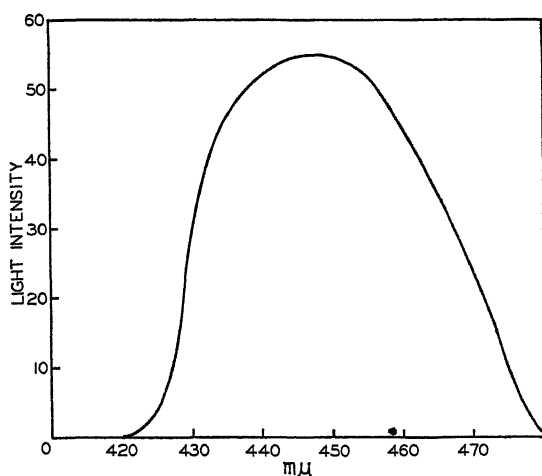


FIG. 5. Intensity distribution (in arbitrary units) of the light emitted by the Cu-carbon anode after the rest of the Cu spectrum had been filtered out.

TABLE VI  
*Estimation of Height of Soret or  $\gamma$ -Band of Pasteur Enzyme*

Wave-length <i>mμ</i>	Estimated $\beta_{\lambda}/\beta_{436}$ if $\beta_{450}/\beta_{436} = 4$	Fraction of intensity contributed	Contribution* to $\beta_{425-430}/\beta_{436}$
425-430	0.75	0.042	0.03
430-435	0.87	0.097	0.08
435-440	1.57	0.121	0.19
440-445	2.85	0.131	0.37
445-450	3.75	0.136	0.51
450-455	3.60	0.131	0.47
455-460	2.18	0.119	0.26
460-465	0.95	0.098	0.09
465-470	0.65	0.074	0.05
470-475	0.52	0.040	0.02
475-480	0.44	0.009	0.004
Calculated.....			2.07
Observed .....			2.02

\* These values are the products of the second and third columns.

determined experimentally. If a broader and more blunt shape is assumed, the  $\beta$  value for  $\lambda = 450$  m $\mu$  will of course be lower.

*Direct Spectrography*

The photochemical experiments indicate that in retina the Pasteur enzyme-CO compound has a steep absorption band at  $450\text{ m}\mu$  and two secondary maxima at  $515$  and  $578\text{ m}\mu$ . A direct spectrographic examination was also performed on rat retinas.

From a transparent plastic (Plexiglas) an absorption cell was constructed which allowed one to compress fourteen rat retinas into a cylinder of  $20\text{ sq. mm.}$  cross-section and  $2\text{ mm.}$  thick. Before examination the retinas in Ringer-glucose solution were treated with  $\text{N}_2$  or with CO. A  $300\text{ watt}$  tungsten biplane filament projection lamp served as the light source. The light was concentrated on the absorption cell which was placed in front of the slit of a small Hilger quartz spectrograph. The small dispersion in the visible region ( $400$  to  $625\text{ m}\mu = 10\text{ mm.}$ ) facilitates the recording of faint absorption bands. For photography in the blue region Eastman Process and No. 40 plates were used; Eastman No. III-B plates served for the study of the entire visible region.

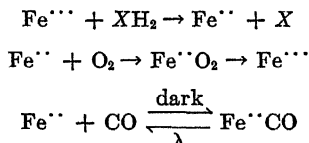
After  $\text{N}_2$  treatment there could be seen on the negatives the cytochrome  $\alpha$ -bands at  $605$ ,  $565$ , and  $550\text{ m}\mu$ , and bands at  $465$  to  $490$  and  $410$  to  $430\text{ m}\mu$ . After CO treatment an additional faint, narrow band appears at  $450\text{ m}\mu$  which coincides in position with the main band of the Pasteur enzyme-CO compound, as revealed by the photochemical experiments.

*Theory*

The Pasteur enzyme contains iron which during the catalysis may exist alternately in the ferric and the ferrous forms. The *ferrous* form is autoxidizable; *i.e.*, it is capable of reacting spontaneously with molecular oxygen to yield the ferric form, possibly via an oxygenated, short-lived intermediate analogous to oxyhemoglobin. The *ferric* form inhibits fermentation, probably by maintaining a component of the glycolytic system in the oxidized, inactive state. The magnitude of the Pasteur effect in a given cell will depend on the *concentration* of the Pasteur enzyme, provided that it operates under conditions in which it is saturated with respect to substrate.

Carbon monoxide competes with oxygen for the ferrous form of the Pasteur enzyme. The distribution of the iron of the enzyme between the two gases is determined by the ratio of their partial pressures and by their affinity to the metal in its particular linkage.

At a CO:O<sub>2</sub> ratio of 8.5 the distribution is strongly in favor of the catalytically inactive FeCO complex. Since the iron participates in a catalytic reaction, no true equilibria but only stationary states may be expected to exist in the cell. The stationary concentration of ferrous iron will primarily depend on the rate of reduction of the ferric form by the substrate (X). The latter may be a dihydrocoenzyme, a thiol, or a metal. Upon illumination, a certain fraction of the iron-carbonyl complex is dissociated, thus making this iron again available for combination with oxygen which is necessary for the suppression of glycolysis. However, it is possible that the *absolute* amount of molecular oxygen so required is very small, owing to a slow or negligible rate of reduction of X by the fermentation system in the presence of air.



This theory explains the inhibition of the Pasteur reaction by CO and its relief by light. The analogy with the theory as developed by Warburg (6) for the respiratory ferment is obvious.

#### DISCUSSION

Following their classical work on the photochemical spectrum of the respiratory ferment in yeast and acetic acid bacteria, Warburg and Negelein, in 1929, reported some experiments on the respiratory ferment in retina (8). The effect of three lines of the mercury arc, *viz.* 405, 436, and 546 m $\mu$ , on the metabolism of CO-treated rat retinas was determined, the rate of *aerobic glycolysis* being used as a "technically convenient" indicator of the activity of the *respiratory ferment*. On the assumption of an intrinsic coupling of respiration with fermentation, an increase in glycolysis was used as a measure of a corresponding decrease in respiration and *vice versa*. The photoefficiency ratios thus determined were compared with those obtained in previous work with microorganisms and hemoglobin.

	$\beta_{405}$ 436	$\beta_{546}$ 436
Respiratory ferment in yeast.....	0.25	0.07
"      "      retina.....	0.32	0.11
Hemoglobin.....	2.0	0.36

From the  $\beta_{\lambda}/\beta_{436}$  ratios obtained with 405 and 546  $m\mu$ , it was concluded that the respiratory ferment of animals possessing hemoglobin is not more closely related to hemoglobin than the respiratory ferment of yeast. There the matter rested until Laser showed, in 1937, that the respiration of retina is not affected by the CO concentration employed by Warburg and Negelein. It is now evident that these workers have actually determined three points on the photochemical spectrum of the Pasteur enzyme.

*Chemical Nature of Pasteur Enzyme*—The pattern of the spectrum of the Pasteur enzyme as it emerges from the present experiments (Fig. 4) indicates that its light-absorbing and CO-binding part is a hemin group. Particularly significant in this respect is the presence of a steep absorption band in the blue region and the existence of two secondary maxima in the blue-green and yellow regions. The thermolabile nature of the enzyme (*cf.* (5)) suggests that the hemin grouping depends for its activity on its combination with a specific protein. The enzyme appears to be linked to the cell structure, since mechanical and chemical injury of the cell tends to abolish the Pasteur effect. The rate of fermentation in cell-free extracts is not affected by oxygen (12).

The location of the  $\alpha$ -band of the enzyme-CO complex in the yellow region, at 578  $m\mu$ , would suggest that its prosthetic group is a *pheohemin*. However, the proximity of the corresponding band of carboxyhemoglobin renders this classification tentative. It finds some support in the fact that the respiratory ferments of yeast and acetic acid bacteria, which also catalyze the reaction of oxygen with suitable substrates, are pheohemin derivatives (13), whereas hemoglobin, which contains a "red" hemin, is catalytically inactive. If the positions of the absorption maxima of the CO complexes of these substances in the visible region are compared (Fig. 6), it becomes evident that no pair of them can be identical.

*Gas Affinities*—Iron porphyrin proteins may be characterized not only by their spectra but also by their affinities for oxygen and carbon monoxide. These affinities, expressed as the dissociation constants,  $K$ , of the complexes, may be derived either from direct gasometric measurements or from the variation of the rate of reactions catalyzed by the hemin derivatives with the partial pressures and concentration ratios of the gases. Accurate  $K$

values are not as yet available for the Pasteur enzyme. Nor has it been possible to demonstrate an inhibition of the respiratory ferment in retina by either CO or low concentrations of O<sub>2</sub>. However, the data presented by Laser (3, 10) permit the assignment of *limiting* values to these constants. A comparison with the corresponding constants as given for the respiratory ferment in yeast and hemoglobin by Warburg and Negelein (11) affords further proof for the non-identity of the various heme proteins (Table VII).

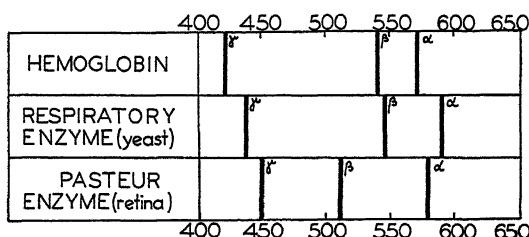


FIG. 6. Absorption maxima of the CO compounds of hemoglobin, the respiratory enzyme in yeast (Warburg), and the Pasteur enzyme in retina.

TABLE VII

*Gas Affinities and Distribution Ratios between CO and O<sub>2</sub> of Some Iron Porphyrin Proteins*

	$\frac{\text{FeO}_2}{\text{FeCO}} \cdot \frac{\text{CO}}{\text{O}_2}$	$\frac{\text{FeO}_2}{\text{Fe} \cdot \text{PO}_2}$	$\frac{\text{FeCO}}{\text{Fe} \cdot \text{PCO}}$
Respiratory ferment (yeast).....	9	$\approx 2500$	$\approx 280$
“ “ (retina).....	$\sim 830$	$\approx 1000$	$\sim 1$
Pasteur enzyme (retina).....	$\sim 0.5$	$\approx 7$	$\sim 10$
Hemoglobin... ..	$\sim 0.01$	$\sim 50$	$\sim 5000$

The values for the Pasteur enzyme listed in Table VII have been obtained on the following assumptions. (1) The aerobic glycolysis in retina is due to an insufficient concentration of Pasteur enzyme; (2) the level of aerobic glycolysis, as observed in oxygen or air, corresponds to the full activity of the enzyme; (3) zero activity exists if the aerobic glycolysis equals the anaerobic glycolysis.

Table VII shows that the affinity for oxygen increases in the following order: Pasteur enzyme (retina)  $\rightarrow$  hemoglobin  $\rightarrow$  respira-



tory ferment (retina)  $\rightarrow$  respiratory ferment (yeast), while the affinity for carbon monoxide increases in the series respiratory ferment (retina)  $\rightarrow$  Pasteur enzyme (retina)  $\rightarrow$  respiratory ferment (yeast)  $\rightarrow$  hemoglobin.

No direct information is as yet available concerning the nature of the respiratory ferment in retina. It is fully active at  $O_2$  concentrations as low as 5 per cent (10), it is not inhibited by CO concentrations as high as 85 per cent, and it is not affected by 0.01 M HCN when the tissue is examined in a bicarbonate medium<sup>3</sup> (3, 14). While it shares the first two features with the respiratory catalysts in certain mammalian tissues (3, 10), it appears to be unique with regard to its cyanide-insensitivity in bicarbonate solution. Furthermore, these three criteria permit a clear cut differentiation between respiratory ferment and Pasteur enzyme in retina, since the Pasteur reaction is strongly inhibited under the same conditions (3, 10, 14).

#### SUMMARY

The *Pasteur enzyme* is defined as the heavy metal-containing, thermolabile agent which catalyzes the inhibition of fermentation by molecular oxygen, a phenomenon known as the *Pasteur reaction*. The spectrum of the enzyme in rat retina has been charted by Warburg's photochemical method. The photochemical efficiency ratios for twenty-one different wave-lengths of light, between 405 and 655  $m\mu$ , have been determined, the mercury line at 436  $m\mu$  being used as the reference standard. The *relative* photochemical spectrum of the Pasteur enzyme-CO complex shows a main absorption band with a maximum near 450  $m\mu$ . Two secondary maxima are located at 515 and 578  $m\mu$ . As compared with the spectrum of the respiratory ferment in yeast or *Acetobacter*, the  $\gamma$ -band of the Pasteur enzyme shows a red shift of about 150 Å., whereas the  $\alpha$ -band shows a corresponding blue shift of about 140 Å. Although the Pasteur enzyme in retina differs from the respiratory ferments of the same tissue, of yeast, and of *Acetobacter* by its affinity for oxygen and carbon monoxide, the general pattern of its spectrum reveals it to be a *pheohemîn proteid* like the respiratory ferments in yeast and *Acetobacter*, the worm

<sup>3</sup> In phosphate media, however, HCN will inhibit the respiration of retina (14).

blood pigment chlorocruorin, and certain cytochrome *a* components.

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# A STUDY OF PURIFIED VIRUSES WITH THE ELECTRON MICROSCOPE

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PLATES 1 TO 4

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Viruses were discovered in 1892 when Iwanowski (1) observed that the agent causing the mosaic disease of tobacco passed through a filter which retained all of the bacteria then known. During the ensuing years many viruses causing diseases in plants, animals, and bacteria have been discovered, and in general these agents have also been found to pass filters which retain ordinary bacteria. It has been necessary, therefore, to devise special means for determining the sizes of these very small infectious agents. For some years the method of ultrafiltration analysis with graded collodion membranes was widely and successfully used (2). Ultra-violet light photography (3), fluorescent microscopy (4), and special staining techniques (5) were also used for some of the larger viruses. By means of such methods it was established that the sizes of viruses ranged from about 250  $m\mu$  down to about 10  $m\mu$ . Although objects as small as 5  $m\mu$  may be rendered visible by dark-field illumination, nothing may be gleaned as to their detailed structure; hence, it is obvious that ordinary microscopy cannot, in general, be used successfully for the viruses, since the limit of resolution for visual light is about 250  $m\mu$ .

During the past 5 years, several viruses have been obtained in highly concentrated and presumably essentially pure form, and it has been possible to learn something of the size and shape of the particles in these preparations by means of sedimentation, diffusion, double refraction of flow, viscosity, and x-ray studies (6).

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One of the outstanding developments was the finding that, in confirmation of an earlier indication (7), some of the viruses depart markedly from a spherical form. For example, by means of data obtained in the studies mentioned above, tobacco mosaic virus was estimated by indirect methods to be about 12  $m\mu$  in diameter and about 400  $m\mu$  in length and to have a molecular weight of about 40 millions (8). Although the theory of the physico-chemical behavior of spherical particles has been worked out fairly thoroughly and appears to rest on a firm foundation, that of particles which depart markedly from a spherical form assuredly is on a much less firm basis. Frampton, for example, considers that in the case of tobacco mosaic virus the asymmetry and molecular weight values calculated from physicochemical data are wholly ambiguous and has shown that by his method of calculation a molecular weight value of infinity on the one hand and of zero on the other hand may be obtained (9). For reasons which have already been discussed (6), there appears to be little justification for the assumption of such an extreme view-point. Nevertheless, the indirect methods provide at best only approximations of the true sizes and shapes of asymmetrical particles and a means for the direct mensuration of such particles has been lacking.

In recent years electron microscopes having resolving powers extending down to about 5  $m\mu$  have been developed. Complete descriptions of the different instruments and of the mode of operation and preparation of specimens may be found elsewhere (10-13). Although excellent micrographs of bacteria have been obtained by means of this apparatus and have proved of value in supplementing information already available (11, 14), it would appear that the electron microscope will be of greatest value in the microscopy of objects having sizes between about 5 and 250  $m\mu$ , a range not covered by the light microscope and one in which practically all viruses have been found to fall. The electron microscope offers the possibility of securing micrographs of individual virus particles and thus of establishing their sizes and shapes with some precision. It should also be possible to determine the extent of the variation in the size and shape of a given virus and even perhaps learn something of the mechanism by means of which a virus particle is duplicated within the host and of the

nature of the difference between strains of a given virus. In this paper are presented the results of preliminary electron microscopic studies of five plant viruses.

#### EXPERIMENTAL

*Tobacco Mosaic Virus*—Tobacco mosaic is the only one of the viruses used in the present study which has been investigated previously by means of an electron microscope (15-18). Most of the virus preparations used in the earlier studies were purified by chemical treatment and such treatment has been found to cause inactivation and aggregation of this virus. Tobacco mosaic virus purified by means of differential centrifugation has been found to be essentially the same as the virus in the untreated infectious juice with respect to biological activity and physicochemical properties (19); hence it appeared desirable to repeat and extend the electron microscope studies with virus purified by differential centrifugation. In preliminary work a small drop of a solution containing 0.2 mg. of four times ultracentrifuged tobacco mosaic virus per cc. in distilled water was applied by means of a capillary pipette to a collodion film about 15 m $\mu$  thick supported on a copper gauze. An attempt was made to secure as thin a film of liquid as possible on the mount. The film was allowed to dry, the mount was placed in the microscope, and the chamber was evacuated. When an area near the center of the mount was brought into focus, the field shown in Fig. 1 (Plate 1) was obtained. It is obvious that the concentration of virus was too great to give a good definition of the individual particles. Fig. 2 presents the appearance of a field nearer the edge of the same mount, in which the individual particles may be seen. Still greater separation of the particles was obtained in an area near the edge, which is shown in Fig. 3. A similar area of another virus preparation which was applied in the same way but at a concentration of 0.01 mg. per cc. is given in Fig. 4 (Plate 2).

The virus shown in Figs. 1 to 3 was used about 3 weeks after preparation. It seemed possible that the granular background and the suggestion of a granular structure for the rods might result from some aging process. Freshly prepared samples of tobacco mosaic virus were applied at a concentration of 0.01 mg. per cc. and examined by means of the electron microscope. Some

of the results which were obtained are shown in Figs. 5 to 7 (Plates 2 and 3), and it may be seen that the granular appearance is absent in these micrographs. However, when a drop of dilute ammonia was added to 1 cc. of an aqueous solution containing 0.01 mg. of tobacco mosaic virus and the preparation immediately observed, the results shown in Figs. 8 and 9 were obtained. The rod-like particles begin to disintegrate with the formation of material which has a granular appearance. It is known from previous chemical work that an excess of alkali causes the denaturation and disintegration of tobacco mosaic virus (20). In Fig. 8 some of the rods may still be seen, whereas in Fig. 9 the field is free of rods and only a granular material remains. However, it is known from previous studies (11) that the film obtained from a dilute solution of an inorganic salt also has a granular appearance. The induced or the spontaneous disintegration of a virus preparation or the presence of a small amount of inorganic material may, therefore, be responsible for the presence of granules. Later micrographs of virus aged for a period of some weeks were similar to those shown in Figs. 5 to 7; hence a granular appearance does not appear to be an invariable result of aging.

Figs. 2 to 7 demonstrate unequivocally the existence of discrete rod-like units in purified preparations of tobacco mosaic virus. The fact that the bulk of the material exists in this form, together with the fact that a great mass of evidence has been accumulated which indicates that the virus activity is associated with such a unit (6), makes it reasonable to assume that the predominating unit shown in Figs. 3 to 6 represents a single particle of tobacco mosaic virus. During the past few years, indirect evidence was obtained which indicated that under certain conditions there occurred an end-to-end as well as a side-to-side aggregation of tobacco mosaic virus (21). Figs. 3 to 6 provide convincing evidence for the existence of elongated aggregates presumably formed by the end-to-end combination of two or more units. Side-to-side aggregation, as well as a combination of this with end-to-end aggregation, is shown in Figs. 5 to 7. The type of aggregation shown in Fig. 7 appears to be that which obtains in the structures which have been referred to as crystals of tobacco mosaic virus (20). There is little indication of a regular structure and, in accordance with earlier results (16), the mass has more

nearly the appearance of a fiber. However, x-ray data have been obtained which indicate that in such aggregates the rods are arranged laterally in two-dimensional, hexagonal close packing (22).

The nature of the forces involved in the end-to-end type of aggregation is of some interest. There is evidence that the ultimate unit of tobacco mosaic virus possesses a dipole moment in the direction of the long axis or that such a moment is induced by an electrical field (23, 24). However, an unsymmetrical distribution of specific charges may be responsible for the marked tendency of the particles to aggregate. The aggregates do not appear to represent the natural form of the virus, for when carefully prepared samples of virus or virus in the freshly expressed untreated infectious juice are examined by means of the analytical ultracentrifuge no evidence for the existence of the aggregates is obtained, whereas following treatment with salt the same samples show either a second sedimenting boundary, presumably due to a component formed by the end-to-end aggregation of two particles, or a more rapidly sedimenting diffuse boundary indicative of even more extensive aggregation. Furthermore, recent micrographs show clearly the unaggregated rods in the freshly expressed untreated juice from mosaic-diseased plants (18). It seems likely that much of the aggregation shown in Figs. 5 to 7 takes place at the time of the drying of the films, when a marked concentration of the virus occurs. As the final stages of the drying occur, a violent whipping motion has been observed by means of an ordinary light microscope. This may be responsible for the formation near the edge of the collodion film of such great masses as those shown in Figs. 5 and 6. The possibility of avoiding aggregation due to such causes through the use of more dilute solutions of virus is being investigated. However, the micrographs already obtained provide good evidence for the existence of a predominating unit having a fairly uniform size and shape. Many measurements of the dimensions of the unit seen in these micrographs have been made and the particle appearing in greatest preponderance is about  $15\text{ m}\mu$  in diameter and about  $280\text{ m}\mu$  in length. The precision in the measurements of particle lengths in this work is of the order of  $5\text{ m}\mu$ , while the absolute error in magnification may be as great as 10 per cent. As may be seen from Fig. 10, measure-



ments of the lengths of all of the 58 particles in two selected fields indicate that a unit having a length of  $280\text{ m}\mu$  predominates. It may be calculated that on a weight basis over 50 per cent of the material exists in the form of particles having a length of  $280\text{ m}\mu$  and over 70 per cent in the form of particles having lengths within 7 per cent of this value. The values for the dimensions of this unit do not conflict with estimates based on x-ray data (22) of a particle diameter of  $15\text{ m}\mu$  and a particle length of some value

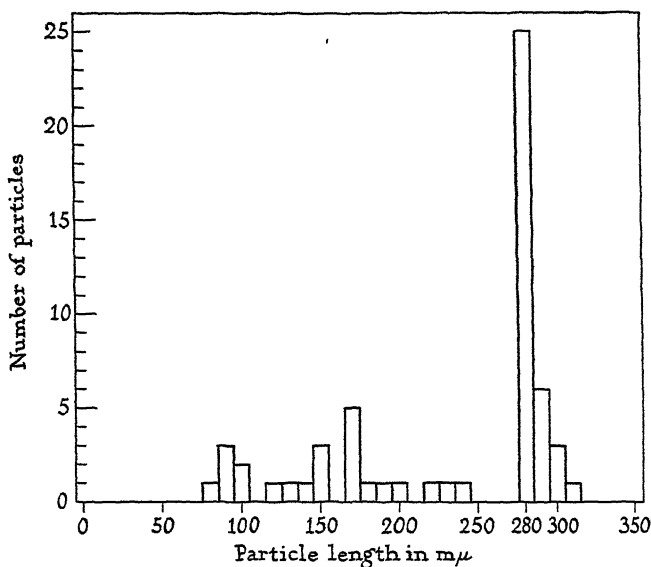


FIG. 10. Distribution of lengths of particles in an ultracentrifugally prepared sample of tobacco mosaic virus.

greater than  $150\text{ m}\mu$ . It seems likely that the value of  $15\text{ m}\mu$  estimated from x-ray data and that of  $280\text{ m}\mu$  estimated from the present micrographs represent the best values for the dimensions of the virus used in the present work. The density of tobacco mosaic virus has been found to be 1.33 (23, 25). The molecular weight of a particle having a circular cross-section  $15\text{ m}\mu$  in diameter, a length of  $280\text{ m}\mu$ , and a density of 1.33 would be  $39.8 \times 10^6$ . This value is in unusually good agreement with the value of  $42.6 \times 10^6$  which was estimated by indirect methods and used tentatively in earlier calculations (21). It has been suggested

that the molecule collapses on drying so that the cross-section is elliptical rather than circular and that the molecular weight is actually slightly lower than the above. However, x-ray data on dried films indicate that there is no extensive collapse of the particles, for there is no distortion of the intramolecular structure and the interparticle distance in such films is  $150\text{ m}\mu$  (22). In addition, it may be possible to secure further evidence by refined techniques designed to determine the molecular weight from the total electron scattering produced by the molecule. However, it is apparent from the present micrographs that tobacco mosaic virus has a definite size, shape, and molecular weight and that the dimensions indicated by the electron microscope studies are of the same order of magnitude as those indicated previously by indirect methods (21). This finding is of importance in connection with the theories of the physicochemical behavior of asymmetrical particles, for it indicates that the indirect methods based on physicochemical data are reasonably valid when correctly used. Tobacco mosaic virus has therefore been of considerable value in demonstrating the usefulness of different methods of approach in the estimation of the size and shape of colloidal particles.

The particle length of about  $280\text{ m}\mu$  indicated by the micrographs of the ultracentrifugally purified tobacco mosaic virus used in the present work is significantly larger than the values of about  $140$  and  $190\text{ m}\mu$  which were estimated by Melchers and coworkers (18) from electron micrographs of two strains of tobacco mosaic virus, one of which is referred to as tomato mosaic virus since it was first noted in tomato plants. These results indicate that the strains of a virus have different particle lengths. Some years ago it was found in this laboratory that the sedimentation constants of different samples of the same strain of tobacco mosaic virus prepared from different lots of the same as well as different species of diseased plants were the same, whereas preparations of strains of tobacco mosaic virus even when obtained from the same type of host plant were found to have different sedimentation constants (26, 27). For example, when determined under the same conditions the sedimentation constant of the strain known as aucuba mosaic virus was found to be about 6 per cent larger than that of ordinary tobacco mosaic virus. Although at that time it was impossible to assign a definite reason for this difference, it was inferred that the difference was due either to a difference in

weight or to a difference in asymmetry. Because of the electron micrographs and the x-ray data which are now available, it seems likely that the particles of strains of tobacco mosaic virus differ both in weight and in asymmetry. The best estimate of particle thickness is probably provided by the x-ray data which indicate that the three strains, ordinary tobacco mosaic, aucuba mosaic, and enation mosaic viruses, all have the same diameter; namely,  $15\text{ m}\mu$ . The electron micrographs show the two strains of virus used by Melchers and coworkers (18) to have particle lengths of about 140 and  $190\text{ m}\mu$ , respectively, and the strain used in the present work to have a particle length of about  $280\text{ m}\mu$ . It is of interest to correlate these dimensions with the sedimentation constants of these preparations. Unfortunately, it is not known whether the sedimentation constant values reported by Melchers and coworkers represent true and reproducible values, since there was no indication of repeated determinations. However, assuming these values to be correct, it follows from Lauffer's work (28) that the values which were each reported to be  $180 \times 10^{-13}$  cannot in fact be identical, for one constant was determined at a virus concentration of 2 mg. per cc., whereas a concentration of 3 mg. per cc. was used for the other. If it be assumed that the variation in sedimentation constant with concentration is similar to that which Lauffer found to obtain with his preparations of tobacco mosaic virus, it may be calculated that the sedimentation constant of  $180 \times 10^{-13}$  at a concentration of 3 mg. per cc. corresponds to a constant of  $183 \times 10^{-13}$  at a concentration of 2 mg. per cc. It may or may not be significant that the corrected value of  $183 \times 10^{-13}$  belongs to the strain having the longer particle length of  $190\text{ m}\mu$ . Although the values which Melchers and coworkers reported for the sedimentation constants may be fortuitous, the results now available for strains of virus at a concentration of 2 mg. per cc. would indicate a correlation between the length of particle and sedimentation constant, for preparations having particle lengths of 140, 190, and  $280\text{ m}\mu$  have sedimentation constants of 180, 183, and  $187 \times 10^{-13}$ , respectively. If these results are treated in the manner described by Lauffer and Stanley (21), it may be seen that there is a good correlation and that it is in accord with theory. Similar calculations show that the sedimentation constant reported for the dimer formed by the end-to-end aggregation of two particles of length  $190\text{ m}\mu$  is in good

agreement with the theoretical value. If the considerations just discussed are valid, it may be predicted that aucuba mosaic virus, which has been reported to have a sedimentation constant about 6 per cent larger than that of the virus used in the present work, should have a particle length of about 330 m $\mu$ . It is obvious, however, that, although the results already obtained indicate that strains of a virus have the same thickness but differ in both weight and particle length, many more experimental data must be obtained before the full significance of the differences between strains may be realized.

More extended observations must be made in connection with the electron microscope studies in order to establish the nature of any artifacts which may result from the drying of the film of a virus preparation or the exposure to the electron beam. The fact that a micrograph taken with the first flow of electrons through a given specimen does not appear to differ from subsequent micrographs taken after longer exposure to the electron beam makes it seem unlikely that gross changes are caused by the electrons. However, the violent motion which takes place as the film dries or the extreme desiccation which occurs on evacuation of the chamber containing the mount may cause some alteration of the specimen. Although it seems very unlikely that these could cause any gross changes in the size and shape of the particles, it is to be hoped that more exact information concerning the nature and extent of any change will become available as the work progresses. It has already been pointed out that on intensive drying of films of tobacco mosaic virus the interparticle distance decreases only from 152 to 150 Å. (22), thus indicating but little shrinkage.

In Figs. 3 to 6 of the present paper, a number of particles are in evidence which are definitely shorter than the predominating unit. It is not known whether these short particles occur regularly in preparations of tobacco mosaic virus or are produced at the time the specimen is mounted. It seems unlikely that they are due to an image produced by a particle of ordinary length which is not lying flat, since surface forces would tend to flatten all the molecules. Furthermore, the particles shown in the micrographs have about the same density, a condition which could obtain only if the particles were lying flat so that uniform thicknesses would be traversed by the electrons. There is at present

no evidence either from activity measurements on the supernatant fluids obtained on ultracentrifugation or from measurements by means of the analytical ultracentrifuge for the existence of these particles. They may, however, possess no virus activity or represent but a small fraction of a preparation and hence not be demonstrable by these methods. The true nature and significance of these short particles is not known at present. If it can be proved that they are not an artifact, that they regularly occur in mosaic-diseased cells and do not represent a degradation product, it is conceivable that they may represent partially synthesized virus particles or viable as well as non-viable virus variants. Nothing is known of the mechanism by means of which a virus particle is duplicated, but it is possible that these particles may provide a clue. The evidence at hand provides no definite indication as to whether duplication is preceded by longitudinal growth and lateral division, lateral growth and longitudinal division, growth from a point, or by some cataclysmic event, although the first possibility might appear most reasonable. It is to be hoped, however, that future work will provide some evidence regarding the course of events during the process of duplication of a virus particle.

*Cucumber Mosaic Virus 3*—Cucumber mosaic virus 3 may be regarded as being rather unusual, since it has not been found transmissible to any plants except members of the Cucurbitaceae (29). Most plant viruses do not have such a narrow host range; tobacco mosaic virus, for example, has been transmitted to forty-six different species of plants representing fourteen widely separated families (30, 31). Despite the fact that cucumber mosaic virus 3 will not multiply in plants susceptible to tobacco mosaic virus and the latter cannot be transmitted to cucumber plants, the two viruses have been found to have very similar physical, chemical, and immunological properties (32).<sup>1</sup> Although the x-ray data indicate a particle thickness of 14.6 m $\mu$ , a value which is considered to be significantly smaller than the value of 15 m $\mu$  for tobacco mosaic virus (22), it seems possible that cucumber mosaic virus may have arisen from tobacco mosaic virus through some fortuitous event. It was therefore of interest to determine whether the micrographs of the particles of the cucumber mosaic virus obtained with the electron microscope would

<sup>1</sup> Knight, C. A., unpublished work.

be similar to those of tobacco mosaic virus. The samples of cucumber mosaic virus 3 used were prepared by means of differential centrifugation by Dr. C. A. Knight. It is a pleasure to thank Dr. Knight for making these preparations and those described in the following section available to us.

An aqueous solution containing 0.1 mg. of the virus per cc. was mounted as previously described and the micrograph reproduced in Fig. 11 (Plate 3) was obtained. It is obvious that the virus solution was too concentrated, so it was diluted with 99 volumes of water. The micrograph obtained with the dilute solution is shown in Fig. 12 (Plate 1). It may be seen that this virus has a rod-like form and that the diameter is about the same as that of tobacco mosaic virus but that the end-to-end aggregation appears to be somewhat more marked than in the case of tobacco mosaic virus. It is possible that the latter may be due to the use of a solution at a slightly more acid reaction and this point is now under investigation. It may be seen from Fig. 13 (Plate 3) that the rod-like particles of cucumber mosaic virus form fibrous aggregates similar in appearance to those formed by tobacco mosaic virus. Although more extensive studies will be required to establish definitely the length of the particle, measurements on the micrographs already available indicate that cucumber mosaic virus 3 has a particle length of about 300 m $\mu$ . The micrographs show, therefore, that, in accordance with previously obtained chemical, physical, and serological data, the ultimate unit of cucumber mosaic virus 3 is similar in size and shape to that of tobacco mosaic virus.

*Cucumber Mosaic Virus 4*—The results obtained with an ultracentrifugally isolated preparation of cucumber mosaic virus 4 at a concentration of 0.01 mg. per cc. are reproduced in Figs. 14 and 15 (Plate 4). A marked tendency to aggregate end-to-end is also noteworthy in the case of this virus. The electron micrograph reproduced as Fig. 14 is very similar to that of cucumber mosaic virus 3 shown as Fig. 12. The result was not unexpected, for the two viruses are strains and have been found to have very similar general properties (32).<sup>1</sup> Although several particles of cucumber mosaic virus 4 about 300 m $\mu$  in length are shown, it is not possible to determine from the micrographs now available whether or not the two strains have different particle lengths.

*Tomato Bushy Stunt Virus*—Tomato bushy stunt virus has been

purified by chemical means (33) and by differential centrifugation (34) and obtained in the form of large rhombic dodecahedral crystals. During the course of these studies, evidence was obtained that the virus particles were essentially spherical in shape and had a diameter of about  $26\text{ m}\mu$  (35). An aqueous solution containing 0.01 mg. of ultracentrifugally isolated bushy stunt virus per cc. was mounted and studied by means of the electron microscope. It may be seen from Fig. 16 (Plate 4) that the particles which are shown have essentially the size and shape indicated by other methods. The tendency of the particles to collect along a fold in the collodion membrane may be noted. Since the size of bushy stunt virus has been well established by different independent methods (34-36), the good agreement of the size of the virus estimated from the electron micrograph with that estimated previously by other methods is significant, for it may be regarded as an indication that no gross change in size occurs during the preparation of the mount.

*Tobacco Necrosis Virus*—Pirie and coworkers (37) purified tobacco necrosis virus by chemical methods and reported that crystalline and amorphous preparations having the same specific virus activity had sedimentation constants of  $130 \times 10^{-13}$  and  $58 \times 10^{-13}$ , respectively. However, Price and Wyckoff (38) found a sedimentation constant of  $112 \times 10^{-13}$  for tobacco necrosis virus purified by differential centrifugation. The size of this virus was estimated to be between 13 and  $20\text{ m}\mu$  by ultrafiltration measurements (39) and by means of radiation studies (40). Since the two extreme values for the sedimentation constant might be considered to indicate a size between about 10 and  $30\text{ m}\mu$ , it is obvious that only the order of the magnitude of the size of tobacco necrosis virus is known. In the present study an aqueous solution containing 1 mg. of ultracentrifugally isolated tobacco necrosis virus per cc. was used and the electron micrograph reproduced as Fig. 17 (Plate 4) was obtained. It may be seen that the particles appear to be spherical in shape and have diameters of about  $20\text{ m}\mu$ .

The writers desire to thank Dr. V. K. Zworykin for his interest and encouragement during the course of the work. It is also a pleasure to thank Dr. L. Marton and Mr. J. Hillier for assistance and advice during the preparation of the micrographs shown in the present paper.

## SUMMARY

Purified preparations of five viruses have been studied by means of the electron microscope. The electron micrographs of the ultracentrifugally isolated tobacco mosaic virus used in the present work showed a predominating unit about  $15\text{ m}\mu$  in width and  $280\text{ m}\mu$  in length and presumably representing single particles of this virus, together with aggregates formed by the end-to-end as well as side-to-side aggregation of this unit and a small amount of rods having shorter although variable lengths. The fact that the dimensions of this unit were of the same order of magnitude as those estimated previously by indirect methods based on physico-chemical data indicates that the latter procedures are useful and essentially valid even for asymmetrical particles when correctly used. Since the particle length of the virus used in the present work was significantly greater than those of two strains studied by other workers, it seems likely that strains of a virus may have different particle lengths. The electron micrographs of cucumber mosaic virus 3 and of its related strain cucumber mosaic virus 4 were very similar, showed a marked amount of end-to-end aggregation, and indicated that the ultimate units were similar in size and shape to that of tobacco mosaic virus. In the case of tomato bushy stunt virus, the micrographs showed spherical particles about  $26\text{ m}\mu$  in diameter, whereas with tobacco necrosis virus the results indicated that the particles were essentially spherical and about  $20\text{ m}\mu$  in diameter.

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## EXPLANATION OF PLATES

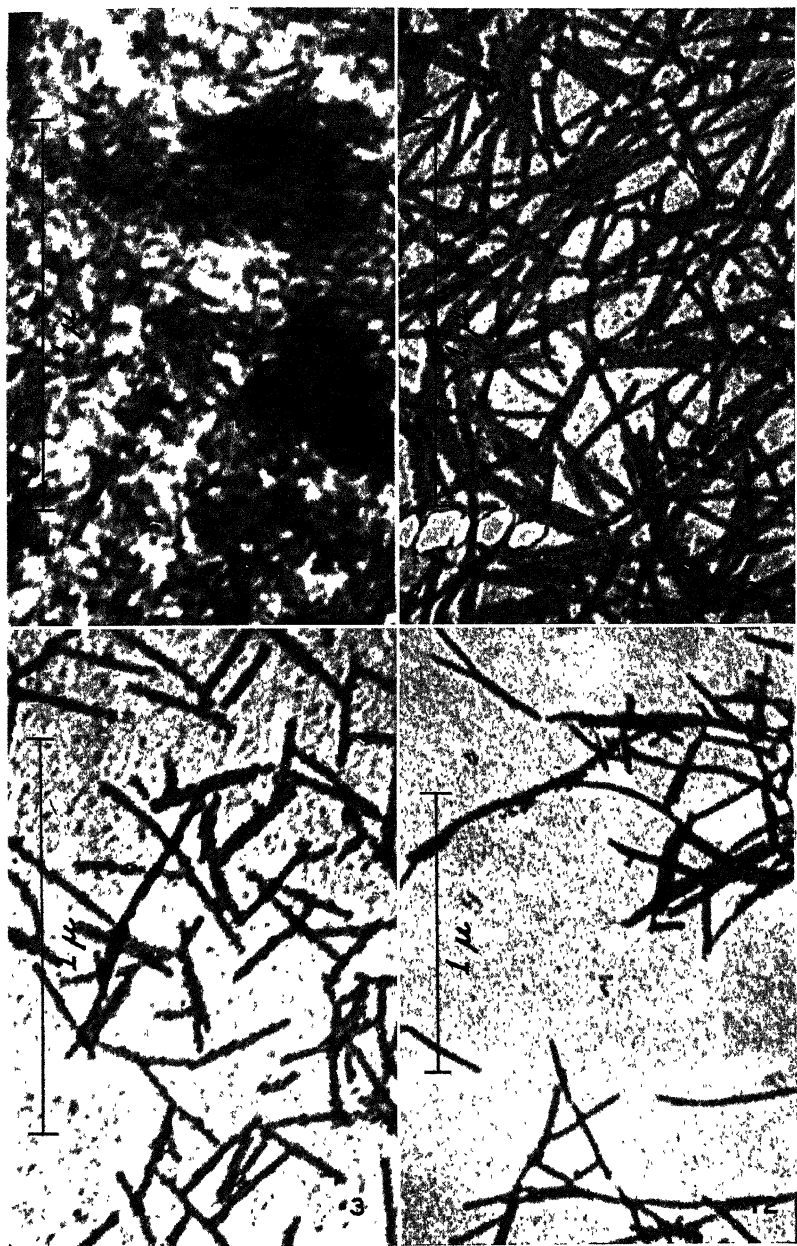
## PLATE 1

FIG. 1. Area near center of mount prepared with an aqueous solution containing 0.2 mg. of ultracentrifugally isolated tobacco mosaic virus per cc.  $\times 55,000$ .

FIG. 2. Area nearer edge of mount used for Fig. 1.  $\times 54,000$ .

FIG. 3. Area near edge of mount used for Fig. 1.  $\times 55,000$ .

FIG. 12. Ultracentrifugally isolated cucumber mosaic virus 3 applied at a concentration of 0.001 mg. per cc., showing single particles and characteristic aggregation.  $\times 39,000$ .

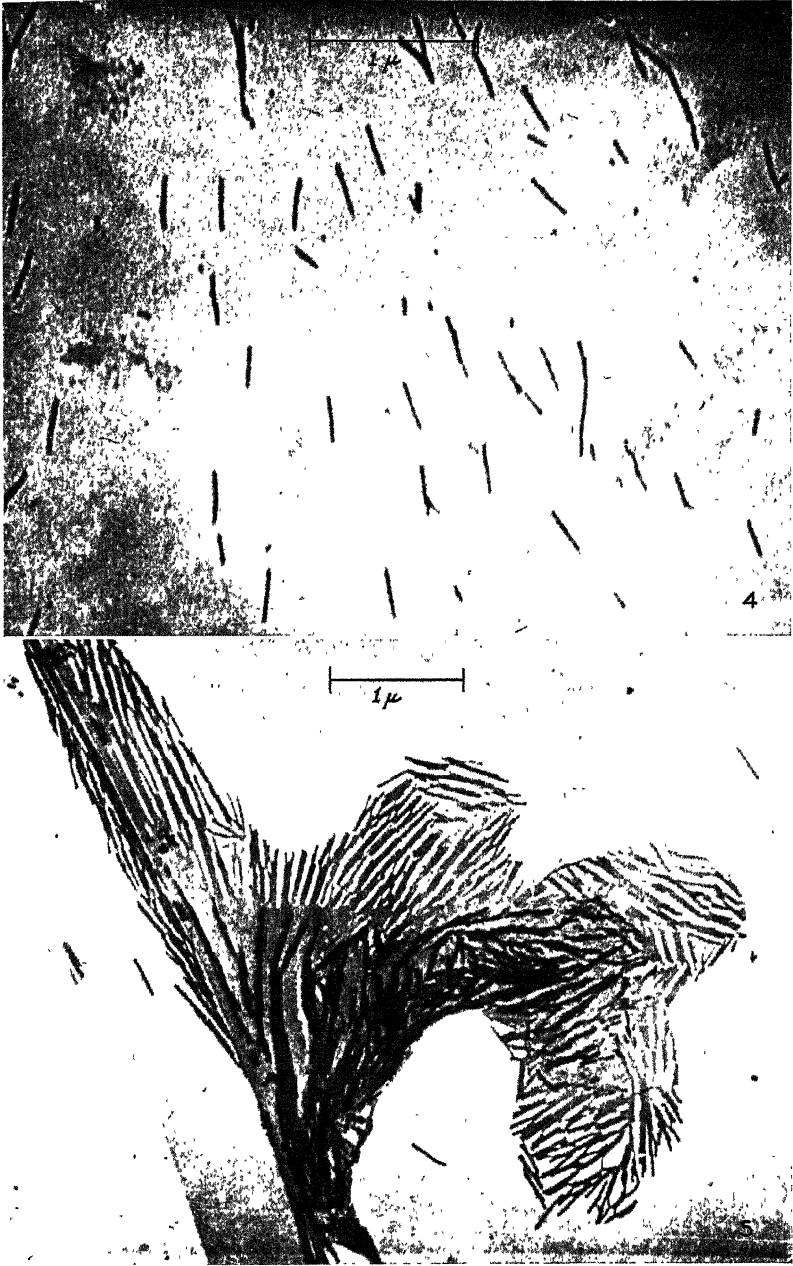


(Stanley and Anderson: Electron microscopy of viruses)

PLATE 2

FIG. 4. Tobacco mosaic virus applied to a collodion film at a concentration of 0.01 mg. per cc.  $\times 24,600$ .

FIG. 5. Tobacco mosaic virus applied at a concentration of 0.01 mg. per cc. Aggregation of particles near the fold in the collodion film may be noted.  $\times 19,500$ .



(Stanley and Anderson: Electron microscopy of viruses)

### PLATE 3

FIG. 6. Aggregation of tobacco mosaic virus near a fold in the collodion film as in Fig. 5.  $\times 17,500$ .

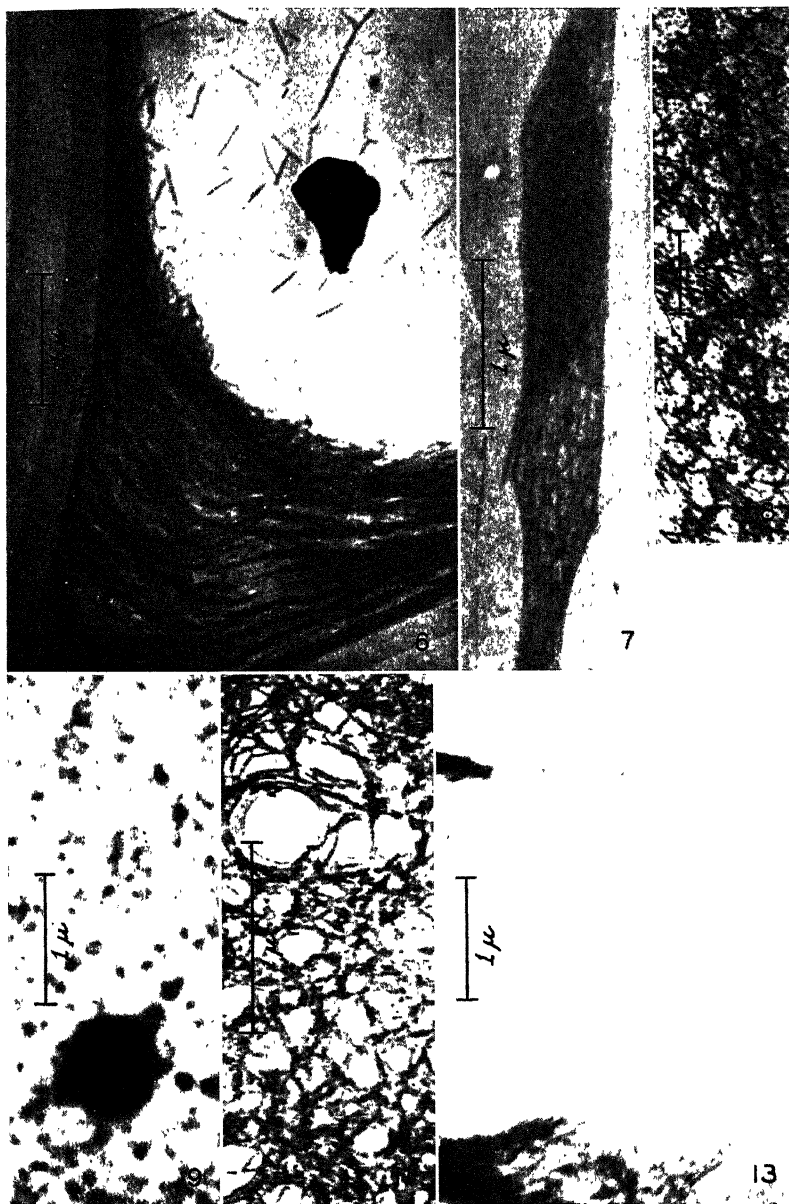
FIG. 7. Fiber-like aggregation of tobacco mosaic virus.  $\times 22,500$ . (Micrograph by Dr. L. Marton.)

FIG. 8. Partial disintegration of tobacco mosaic virus by dilute ammonia.  $\times 11,250$ .

FIG. 9. Complete disintegration of tobacco mosaic virus by dilute ammonia.  $\times 17,500$ .

FIG. 11. Ultracentrifugally isolated cucumber mosaic virus 3 applied at a concentration of 0.1 mg. per cc. A thick mat of virus and holes in the collodion film may be noted.  $\times 25,800$ .

FIG. 13. Fiber-like aggregate of cucumber mosaic virus 3.  $\times 16,700$ .



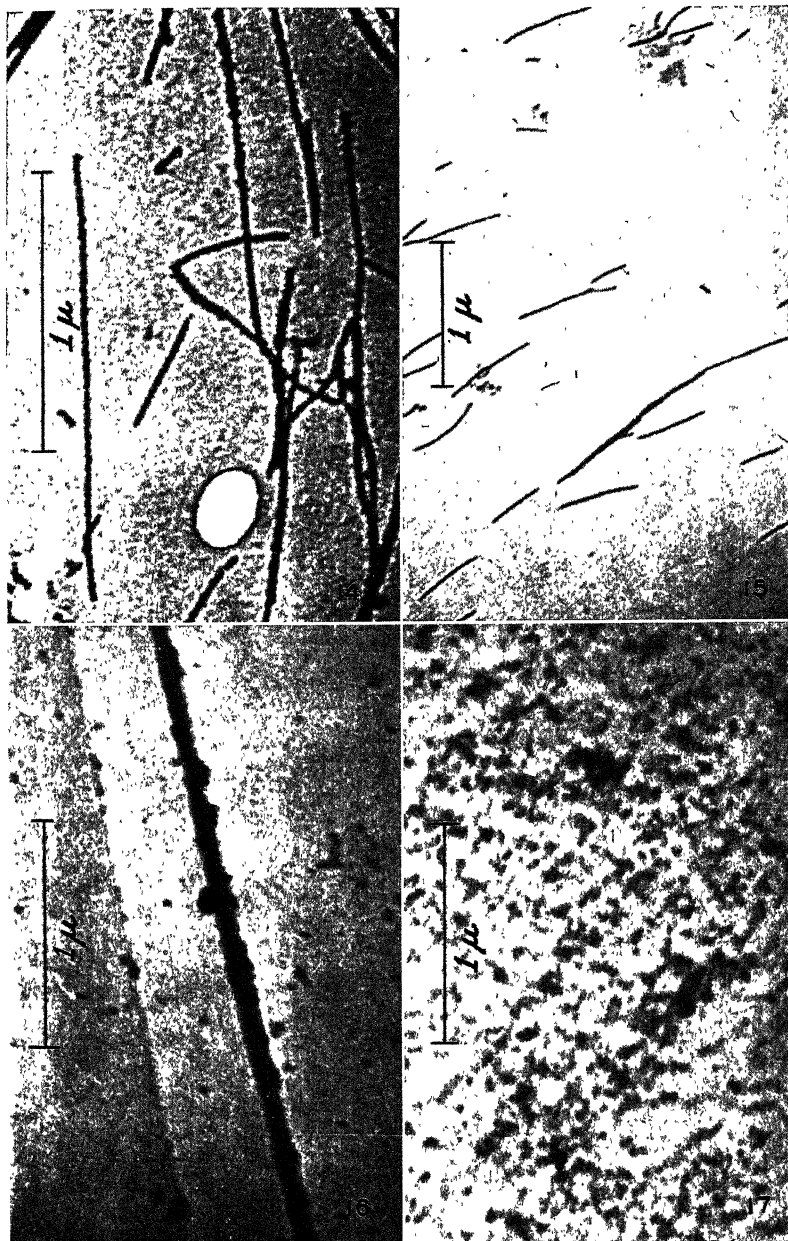
#### PLATE 4

FIG. 14. Cucumber mosaic virus 4 applied at a concentration of 0.01 mg. per cc. End-to-end aggregation of particles is especially noteworthy.  $\times 39,000$ .

FIG. 15. Cucumber mosaic virus 4. Several particles about 300  $m\mu$  in length are shown.  $\times 20,000$ .

FIG. 16. Ultracentrifugally isolated bushy stunt virus applied at a concentration of 0.01 mg. per cc. It may be noted that there is a tendency for the particles to collect at folds in the collodion membrane.  $\times 31,600$ .

FIG. 17. Ultracentrifugally isolated tobacco necrosis virus applied at a concentration of 1 mg. per cc.  $\times 30,500$ . (Micrograph by Dr. L. Marton )



(Stanley and Anderson: Electron microscopy of viruses)





# A STUDY BY MEANS OF THE ELECTRON MICROSCOPE OF THE REACTION BETWEEN TOBACCO MOSAIC VIRUS AND ITS ANTISERUM

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## PLATE 5

(Received for publication, February 3, 1941)

From the results of numerous experiments, immunologists have inferred that when a foreign material, usually protein in nature and called an antigen, is injected into an animal, it induces the formation of substances called antibodies, which appear in the animal's blood serum and which will react with the antigen injected (1-4). When this reaction results in the formation of a precipitate, it is called a "precipitin reaction." It seems likely that 1 antibody molecule is able to attach itself to more than one antigen particle and, *vice versa*, in such a manner that a framework or "lattice" of antigen particles is formed which gives rise to an antigen-antibody precipitate (1, 3, 5). On the basis of these ideas, explanations of the various phenomena of immunity have been advanced.

With the development of electron microscopes (6, 7) capable of recording the sizes, shapes, and reactions of protein molecules (8), it should be possible to observe certain of these immunological reactions directly. There are reported in the present paper the results of a preliminary study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum. Tobacco mosaic virus was selected as the antigen because of its large size and distinctive shape. Many kinds of evidence indicate that the virus particles used in the present work are essentially cylindrical in shape and have lengths of about 280 m $\mu$  and

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diameters of about  $15\text{ m}\mu$  (8, 9). Rabbit antiserum was selected as a source of antibodies to tobacco mosaic virus because the reactions involved have been extensively investigated (10) and because much information is available concerning the antibodies in rabbit sera (11, 12).

#### EXPERIMENTAL

Anti-tobacco mosaic virus rabbit serum was prepared and 1 cc. was added to 1 cc. of a solution containing 1 mg. of tobacco mosaic virus. It seems likely that in such a mixture the reaction would occur either in the equivalence zone or in the region of excess antibody (3, 10). The mixture was shaken and separate portions immediately diluted 1:10 and 1:100 with distilled water. After the preparations had stood for 1 hour at  $25^{\circ}$ , mounts of these solutions for the electron microscope were prepared in the usual manner (8) and electron micrographs were made of them. As a control, parallel preparations of tobacco mosaic virus alone and with added normal rabbit serum were made and micrographs prepared. After several hours a marked precipitate in the undiluted mixture of virus and antibody, a faint precipitate in the 1:10 dilution, and a faint Tyndall effect in the 1:100 dilution were observed. Mounts of these preparations were then made and observed by means of the electron microscope.

In Fig. 1 is presented the micrograph from the suspension containing 0.01 mg. of tobacco mosaic virus per cc. It may be noted that the molecules stand out sharply<sup>1</sup> and have widths of about  $15\text{ m}\mu$  and lengths of about  $280\text{ m}\mu$ .

Fig. 2 is a micrograph of a mixture of tobacco mosaic virus and normal rabbit serum diluted 1:100 with distilled water. Here again the tobacco mosaic virus molecules stand out sharply with the normal lengths and widths, and with but few particles from the serum adsorbed on them. The contaminating bacterium

<sup>1</sup> It is convenient in obtaining micrographs of viruses to focus the electron microscope on relatively large and heavy particles. The pictures of these particles then reveal the order of resolution. This procedure is possible because of the great depth of focus of the electron microscope. Thus, the sharpness of the black particles in Fig. 1, of the bacterium in Fig. 2, of the edge of the collodion membrane in Fig. 3, and of the particle in Fig. 4 attests to the fact that the virus particles in all cases are in sharp focus and that the variations in widths which are observed are real.

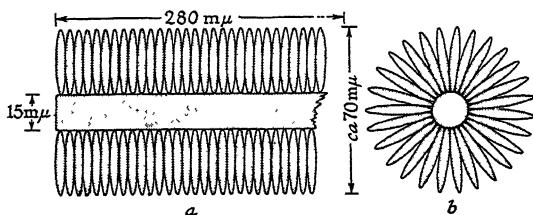
which may be observed in this figure serves to give a good idea of the relative size of the particles of tobacco mosaic virus. It may be seen that some of the tobacco mosaic virus molecules are adsorbed at right angles to the surface at the end of the bacterium. When mixtures of tobacco mosaic virus with precipitating rabbit antisera of approximately equal potency to tomato bushy stunt, potato latent mosaic, and tobacco ring spot viruses were used, similar results were obtained and there was no indication of an increase in the dimensions of the molecules of the tobacco mosaic virus.

Fig. 3 is a micrograph of the mixture of tobacco mosaic virus and antiserum diluted 1:100, which had stood for 1 hour. It is seen at once that the particles of tobacco mosaic virus appear at much greater contrast and are 3 or 4 times as wide as in the former preparations. This indicates that particles from the antiserum have become attached to the tobacco mosaic virus molecules, making them appear wider and at the same time presenting a thicker specimen to the electron beam. It is to be concluded that serum antibody molecules attach themselves at a great many points distributed over the surface of the tobacco mosaic virus molecule.

Fig. 4 shows a typical portion of the preparation of tobacco mosaic virus plus antiserum after standing for several hours at a dilution of 1:100. It may be seen that the particles are still extremely thick and, although the field is in sharp focus,<sup>1</sup> the particles appear fuzzy. Thus, there is a density gradient at the edge of the particles which may be due to the radial distribution of asymmetrical particles on the antigen. It is known that tobacco mosaic virus antibodies produced in rabbits are much smaller than the virus molecules,<sup>2</sup> and it seems likely that their dimensions are approximately the same as those of the antibody globulins of antipneumococcus rabbit sera which are considered to be about  $3.7\text{ m}\mu$  in diameter and  $27.4\text{ m}\mu$  in length (11-13). If a single layer of molecules having approximately these dimensions were to become attached laterally to a molecule of tobacco mosaic virus, the increase in diameter would be only about  $8\text{ m}\mu$  or an amount insufficient to explain the increase in width noted in Fig. 4. If, however, the molecules become attached to the virus molecule

<sup>2</sup> Stanley, W. M., unpublished work.

by their ends in a radial manner similar to that shown in Text-fig. 1, then the increase in width would be of the order of  $55\text{ m}\mu$ , or an amount sufficient to explain the increase in width noted in Fig. 4. It is also obvious from Text-fig. 1 that an electron beam passing through such an aggregate at right angles to the direction of the length would encounter regions of decreasing density as the edge was approached. An arrangement of asymmetrical molecules, such as that shown in Text-fig. 1, could therefore account for the indefinite and fuzzy appearance of the edges of the particles shown in Figs. 3 and 4. Furthermore, such a radial arrangement would be in accord with certain aspects of a recent theory of the structure and reactions of antibodies (5). It may also be seen from Fig. 4 that the antibodies seem to have joined the antigen molecules together to give at least the outward appearance of the



TEXT-FIG. 1. Possible mode of attachment of small asymmetrical shaped molecules to the side surface of a molecule of tobacco mosaic virus. (a) Longitudinal cross-section; (b) lateral cross-section.

framework or "lattice" of immunological theory. The irregularity of the observed framework, if due to such a chemical interlinking of antigen and antibody, would justify the avoidance of the term "lattice" by Heidelberger (3) and by Pauling (5).

Although the virus molecules appear to be well covered, it is not possible to determine from the present micrographs whether or not the coverage is complete. The fact that the dimensions of the virus molecules were unchanged in micrographs of mixtures with antisera to other viruses indicates that the primary reaction under investigation is specific in nature. Whether or not any non-specific serum protein was subsequently adsorbed onto the antigen-antibody complex cannot be determined until purified antibody preparations are used. Confirmatory evidence of the specificity of the primary antigen-antibody reaction was obtained in a study by means of the ultracentrifuge in which it was found

that the sedimentation constant of tobacco mosaic virus was essentially unchanged in mixtures containing an excess of normal rabbit serum or antisera to bushy stunt, ring spot, or latent mosaic viruses.<sup>2</sup> It was, of course, impossible to determine the sedimentation constant in the case of a mixture containing an excess of anti-tobacco mosaic virus serum, for the virus was completely precipitated and only the boundary due to the globulin fraction was apparent. In marked contrast, the sedimentation constant of bushy stunt virus was essentially unchanged in a mixture with anti-tobacco mosaic virus serum. The lack of reaction between bushy stunt virus and anti-tobacco mosaic virus serum was also demonstrated by means of electron micrographs.

The electron microscope thus makes it possible to observe directly the result of the reaction of particles in antiserum with antigen molecules and to record the nature of the precipitate which results from this interaction. The present results demonstrate the usefulness of the asymmetrically shaped tobacco mosaic virus and the advisability of making extensive studies of the antigen-antibody reaction by means of the electron microscope.

The authors are indebted to Dr. Michael Heidelberger, Dr. Stuart Mudd, and Dr. V. K. Zworykin for many helpful suggestions.

#### SUMMARY

1. Electron micrographs of tobacco mosaic virus deposited on a collodion film show that the molecules are about 280  $m\mu$  long and about 15  $m\mu$  wide.

2. Micrographs of a mixture of virus and normal rabbit serum show virus particles of normal size and indicate little or no adsorption of particles from normal serum onto the virus molecules. Similar results were obtained with mixtures of tobacco mosaic virus with antisera to tomato bushy stunt, potato latent mosaic, and tobacco ring spot viruses.

3. A mixture of tobacco mosaic virus and tobacco mosaic virus antiserum from rabbits, when dried on a collodion film an hour after mixing and examined by means of the electron microscope, shows particles about 60  $m\mu$  wide, about 300  $m\mu$  long, and having fuzzy profiles. The increase in particle width and the fuzzy appearance are regarded as indicating that the ends of asymmetri-

cally shaped molecules from the serum react specifically with the antigen molecules. No reaction between anti-tobacco mosaic virus serum and bushy stunt virus was demonstrable.

4. When the mixture of antigen and antiserum is applied to a collodion film several hours after mixing, an irregular framework of thickened antigen molecules may be seen. It is this framework which makes up the antigen-antiserum precipitate. The results demonstrate the usefulness of the electron microscope and of a large and distinctively shaped antigen such as tobacco mosaic virus in the study of the antigen-antibody reaction.

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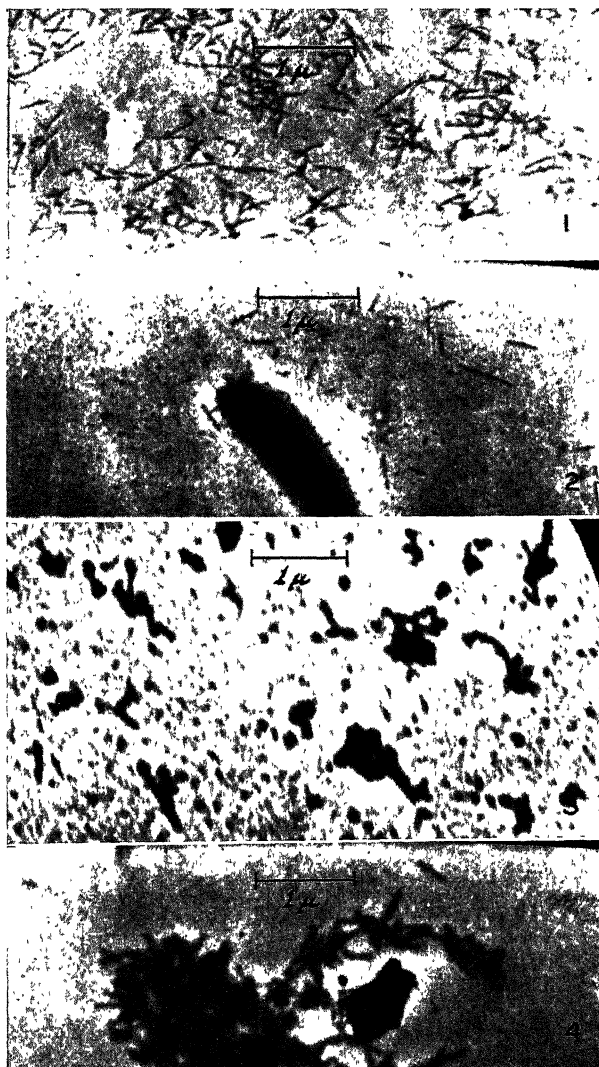
#### EXPLANATION OF PLATE 5

FIG. 1. Ultracentrifugally isolated tobacco mosaic virus applied to a collodion membrane at a concentration of 0.01 mg. per cc., showing particles having a width of about 15  $\mu$ .  $\times 13,700$ .

FIG. 2. Mixture of same tobacco mosaic virus preparation used for Fig. 1 at a concentration of 1 mg. per cc. with an equal volume of normal rabbit serum and applied at a 1:100 dilution. The width of the virus particles is unchanged.  $\times 13,800$ .

FIG. 3. Mixture of same tobacco mosaic virus preparation used for Figs. 1 and 2 with an equal volume of an anti-tobacco mosaic virus rabbit serum and applied at a dilution of 1:100 to the collodion film 1 hour after mixing. Particles appear more dense and are about 60  $\mu$  wide.  $\times 13,200$ .

FIG. 4. Virus-antiserum mixture of Fig. 3 applied at a dilution of 1:100 several hours after mixing. Note that thickened virus particles have fuzzy profiles and have formed an irregular framework.  $\times 13,700$ .



(Anderson and Stanley: Antigen-antibody reaction)





# MONOLAYERS OF COMPOUNDS WITH BRANCHED HYDROCARBON CHAINS

## IV. PHTHIOIC ACID\*

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(Received for publication, February 3, 1941)

Phthioic acid is an optically active, branched chain, saturated acid with the formula  $C_{26}H_{52}O_2$ , which has been isolated from the tubercle bacillus by Anderson and Chargaff (2, 3). The free acid is non-crystalline, melts at  $20-21^\circ$ , and has an optical rotation of  $[\alpha]_D = +12.6^\circ$ . The constitution of this acid is of unusual interest, as it has been shown by Sabin (4) that the purified acid when injected into normal animals produces typical tubercular tissue.

Owing mainly to the difficulty of obtaining the acid in any quantity, it has not yet been possible to elucidate its structure by ordinary methods of organic chemistry.

From a study of the products given by phthioic acid on oxidation and degradation Spielman and Anderson (5) concluded that it possessed a branched chain with methyl groups probably in the  $\alpha$  position and in the neighborhood of carbon atom 11 and that other branches existed, but their number and position could not be determined.

It was thought that a study of the structure of monolayers on water and multilayers on solid surfaces and a comparison of the results with those obtained with compounds of known constitution, studied in the earlier parts of this series (6, 7),<sup>1</sup> might give some

\* A preliminary account of this work was given at a meeting of the Chemical Society (London) at Imperial College, March 2, 1939; cf. Sir Robert Robinson, Presidential Address to the Chemical Society (London), 1940 (1).

<sup>1</sup> Ställberg and Stenhagen, unpublished work.

valuable hints as to the constitution of the phthioic acid. Professor Anderson kindly put a specimen of the pure acid at the disposal of the authors.

For a complete investigation of the mono- and multilayer structure only a few mg. of the substance are needed, which is a great advantage of the method.

### *Monolayers*

*Technique*—The monolayer measurements were carried out with a Langmuir-Adam trough and a single torsion wire surface balance, sensitive to 0.05 dyne. Surface potentials were measured as described by Schulman and Rideal (8) with a polonium electrode and a Compton electrometer (short period pattern, Cambridge Instrument Company). A trough of Pyrex glass, a mica boom, and vaselined silk thread side barriers were employed. Petroleum ether (b.p. 60–80°) was used as a solvent for the acid (about 20 cc. for 5 mg.) and spreading was effected by means of an Agla micrometer syringe. Care was taken that the water used was free from heavy metal ions. The temperature was kept constant to within 1°. On acid and neutral substrates the surface potential was uniform within 5 millivolts over different parts of the monolayer, and the error in the area measurements is within 2 or 3 per cent. On alkaline substrates, the experimental error may be larger.

A number of force-area curves were also obtained with an automatic recording surface balance of the Wilhelmy-Dervichian type (9), built in collaboration with the authors by Mr. K. J. I. Anderson at the Institute of Physical Chemistry, Uppsala.

*Results*—The force-area curves obtained with the acid in undissociated form on 0.01 N HCl substrate are shown in Fig. 1. At a temperature of 5° the limiting area is 52 sq. Å. The monolayer is not very compressible and collapses at an area of 36.5 sq. Å. and a pressure of 10.5 dynes. The monolayer is liquid and a comparison with the results obtained on substrates containing barium ions (see below) shows that it is mesomorphic or "liquid-condensed" at this low temperature. Increase in temperature to 20° gives a general expansion, and the limiting area increases to 62 to 63 sq. Å. The area and pressure at the point of collapse depend somewhat on the rate of compression. This

was well shown by a series of experiments carried out with the automatic Wilhelmy balance. The trough used was  $16 \times 38$  cm., the amount of phthioic acid spread was 0.068 mg., and the monolayer was compressed continuously by a barrier which was moved forward at speeds that could be varied between 2.5 and 38 cm. per minute. The lower curve of Fig. 2 was obtained at the lowest speed and shows an initial flat collapse starting at about 11 dynes

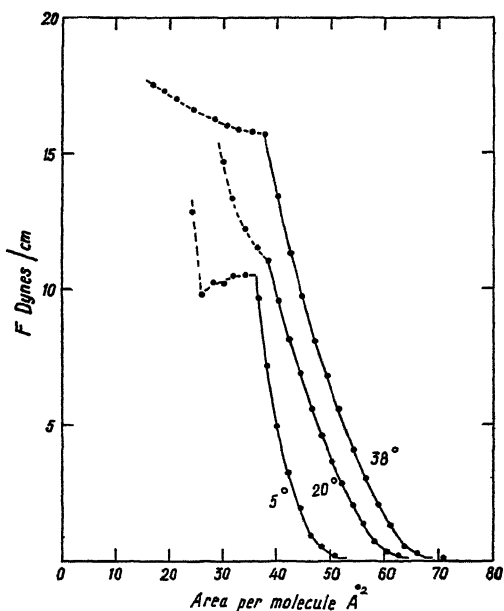


FIG. 1. Force-area curves at different temperatures for phthioic acid spread on acid substrate (0.01 N HCl).

pressure. This is followed by a rise and a new flat part at about 18 dynes. The upper curve was obtained at a speed of 38 cm. per minute. The collapse now sets in at about 13 dynes and is followed by a fall in pressure down to about 10 dynes, which is followed by a new rise and a flat at 18 dynes. The form of this part of the collapse curve is remarkably constant. The lower part of the force-area curve from the limiting area to the point of collapse is identical at both speeds (the slight kink in the beginning of the upper curve is accidental and due to the apparatus). When

compressed beyond the point of collapse, on to the second flat at 18 dynes pressure, the collapsed monolayer has a gel character, and talc particles on the surface show elastic recoil when displaced by gentle blowing. The reproducibility of the force-area curves is very good and they are completely reversible in that the same curve is obtained on expansion and recompression of the monolayer as on the original compression, even if the latter is

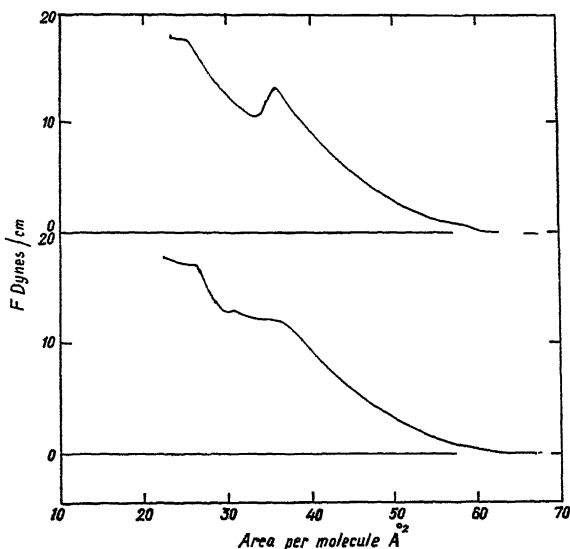


FIG. 2. Photographic reproduction of automatically registered force-area curves for phthioic acid on 0.01 *N* HCl at 20°. Width of trough 16 cm., amount of phthioic acid spread 0.068 mg. Upper curve, rate of compression, 33 cm. per minute; lower curve, 2.5 cm. per minute.

carried far beyond the point of collapse, provided that the expansion is not carried out too rapidly. At 38° (Fig. 1), the monolayer is still more expanded, but stands somewhat higher pressures. At 18 dynes pressure a flat collapse sets in. The different type of collapse at this temperature compared with 20° is probably connected with the fact that the acid is a liquid in three dimensions at 38°, while at 20° it is just on the point of melting.

The surface potential at 20° (Fig. 3) is about 230 millivolts at the limiting area and rises on compression to about 325 millivolts

at the point of collapse. The potentials are uniform to about 5 millivolts over different parts of the surface. The apparent surface dipole moment  $\mu$ , calculated according to the formula  $\Delta V = 4\pi \cdot n \cdot \mu$  where  $\Delta V$  = the observed surface potential and  $n$  = the number of molecules per unit area, is 380 millidebyes at 60 sq. Å. and falls to about 350 at 40 sq. Å. These values are exceptionally high for a saturated aliphatic fatty acid.

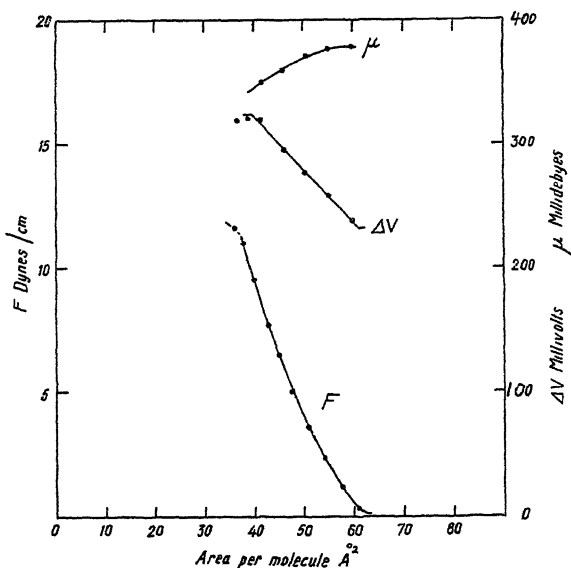


FIG. 3. Force-area, surface potential-area, and apparent surface moment-area curves for undissociated phthioic acid on acid (0.01 N HCl) substrate at 20°.

When spread on a  $M/150$  phosphate substrate, pH 7.2 (Sørensen buffer, diluted ten times, *cf.* (10) p. 210) (Fig. 4), the limiting area at 20° is about 67 sq. Å. and collapse occurs at an area of 36.5 sq. Å. and 18 dynes pressure. Owing to the partly ionized state, the surface potential is about 140 millivolts lower than on the acid substrate.

In the fully ionized state, when spread on 0.01 N NaOH (Fig. 4), phthioic acid, in common with other acids with branched hydrocarbon chains (6), gives a vapor film. The surface potential is

negative at large areas and passes through zero on compression. The reproducibility of the measurements on this substrate is rather poor.

Fig. 5 shows the force-area curves for 6° and 20° obtained on a substrate containing barium ( $\text{BaCl}_2$   $3 \times 10^{-5}$  M,  $\text{KHCO}_3$   $4 \times 10^{-4}$  M) similar to that used for building multilayers. At

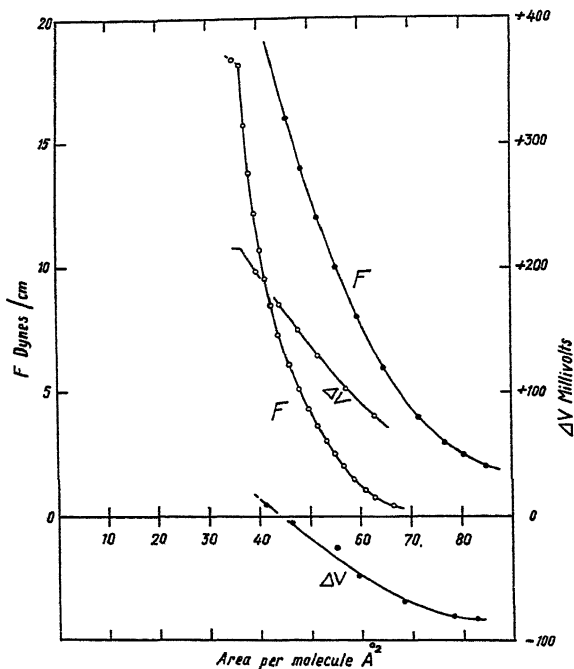


FIG. 4. Force-area and surface potential-area curves (○) for phthioic acid on neutral substrate (M/150 phosphate buffer, pH 7.2); (●) on alkaline substrate (0.01 N NaOH).

6° the monolayer is mesomorphic and the force-area curve is almost identical with the curve at 5° on 0.01 N HCl substrate (Fig. 1). At 20° (Fig. 5) the lower part of the force-area curve is somewhat more expanded. The monolayers collapse readily above 14 dynes pressure (when compressed below an area of 36 to 36.5 sq. Å.). The unstable region is indicated by the dotted line. When compressed beyond the point of collapse, the monolayer turns into a solid "skin" on the water surface.

*Multilayers*

*Technique*—The technique described by Blodgett (11) was used. The acid was spread from a solution in freshly redistilled petroleum ether on a substrate containing  $3 \times 10^{-4}$  M barium chloride and a trace of copper ions (distilled water from an ordinary still). The pH of the substrate was about 6.5 and the multilayers were built on chromium-plated brass slides  $3 \times 12 \times 70$  mm. The slides

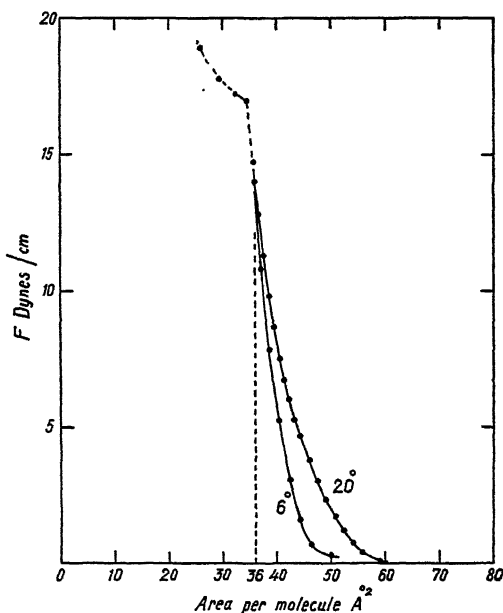


FIG. 5. Force-area curves for phthioic acid on a neutral substrate containing barium ( $\text{BaCl}_2$   $3 \times 10^{-5}$  M,  $\text{KHCO}_3$   $4 \times 10^{-4}$  M) at  $6^\circ$  and  $20^\circ$ . The dotted line indicates the unstable region.

were cleaned by polishing with the finest grade of emery cloth and subsequent rubbing with pure cotton wool under running water.

Castor oil with a spreading pressure of 16 dynes brought about collapse of the monolayers and could not be used as piston oil, but an oxidized motor oil having a spreading pressure of 13 dynes gave good results. The motor oil did not behave as a perfect piston, as the pressure rose appreciably with compression of the piston. This trouble was minimized by using a large piston sur-



face and keeping the piston nearly fully expanded during the building of the multilayers by moving the glass barrier opposite the piston forward after each dip. With this piston Y-deposited films (deposition occurring both on the down and the up journey of the slide) were obtained. At 20° the multilayers were not optically perfect but perfect films could be built if the temperature of the substrate was kept at 5°. The fact that multilayers can be easily built shows that the monolayer on water is mesomorphous (12) and the optical perfection of the films proves that the substance is properly spread on water. This test of the homogeneity of the monolayer is at least as sensitive as the ultramicroscopical examination often employed for the same purpose.

Films containing 100 layers and more could be easily built at 5°. Multilayers of barium phthioate are, compared with multilayers of barium stearate, rather soft. When kept in a refrigerator at temperatures below 0°, the multilayers of barium phthioate are unchanged after several weeks, but at room temperature the films get slightly fogged in about a day.

A similar behavior has been observed for multilayers of long chain esters (13), and in that case it has been shown to be due to the growth of the originally very small crystallites within the multilayer, to sizes comparable with the wave-length of visible light, a process which proceeds at a higher rate with higher temperature.

Owing to the low melting point of the phthioic acid, multilayers of the free acid built from acid substrate cannot be obtained. An equimolecular mixed film of octadecylamine and phthioic acid gave good X-deposited films (deposition on the down journey of the slide only) from a substrate consisting of a phosphate buffer (0.05 M, pH 7.2; Clark and Lubs (10) p. 200). The mixed monolayer could be compressed by a castor oil piston without collapse and the multilayer obtained was quite stable at room temperature.

*Optical Measurements of Thickness per Layer*—The optical thickness per layer for the multilayers of barium phthioate built at a temperature of 5° with a piston pressure of 13 to 13.5 dynes was determined by comparing the interference colors given by the phthioate films with standard color comparison gages of barium stearate, prepared according to the directions of Blodgett (14). The stearic acid used for the standard had been carefully purified

via the ethyl ester and the solvent (petroleum ether) freshly redistilled. The phthioic acid multilayers were built on an initial rubbed down layer of barium stearate. The color comparison was carried out in polarized white light, with the ordinary ray, by viewing the films through a Polaroid screen. Between 40 and 60 phthioate layers were compared with 30 to 46 stearate layers, the comparison being carried out to the nearest layer. The mean of six determinations gave an optical thickness for barium phthioate of  $18.2 \pm 0.5 \text{ \AA}$ ., Blodgett's value  $24.4 \text{ \AA}$ . (15) being taken for the optical thickness per layer for barium stearate and the refractive index for the ordinary ray assumed to be equal in both films. The refractive index for barium phthioate was not determined directly, as suitable glass slides with known refractive indices were not available, but the error introduced by assuming the refractive index of the two multilayers to be equal will probably not be greater than 1 or 2 per cent.

The deposition ratio  $R$  (the ratio of the area occupied by the film on water to the geometrical area occupied on the solid surface) was determined by measuring the area consumed on the water and the area occupied by the multilayer on the metal slide, and was found to be  $R = 0.97$  to  $0.98$ . For barium stearate Langmuir, Schaefer, and Sobotka (16) found  $R = 0.99$ .

#### *x-Ray Measurements on Barium Phthioate Multilayers*

*Technique*—The long x-ray spacing of barium phthioate multilayers deposited on chromium-plated slides and consisting of 40 to 100 Y-deposited layers was measured by filtered copper  $K_{\alpha}$  radiation and also monochromatic copper  $K_{\alpha}$  radiation, obtained from a condensing monochromator of pentaerythritol of the type described by Fankuchen (17). The strictly monochromatic radiation gives considerably less background than the filtered radiation. The slide was mounted vertically in such a way that the axis of the goniometer was lying in the surface of the slide. The direction of dipping was normal to the direction of the incident radiation. The camera distance was 8 to 13 cm. and the measurements were performed in air at room temperature. The slide was oscillated through  $5^{\circ}$ . A fairly sharp diffraction pattern showing four orders of a spacing  $35.0 \pm 0.7 \text{ \AA}$ . was obtained. Odd orders were slightly stronger than the even.

*Interpretation of x-Ray Pattern*—It has been shown that multilayers are microcrystalline and that the long x-ray spacing obtained always corresponds to the long spacing of one of the crystalline forms in which the substance forming the multilayer can exist (13). Y-deposited films of barium salts of fatty acids contain a fairly high percentage of free acid, but the x-ray pattern obtained is that of the barium salt (18).

Phthioic acid itself has not been obtained in crystalline form, but phthioamide has been obtained in the form of microcrystalline leaflets by Spielman and Anderson (5). The multilayer technique is a convenient method for obtaining oriented crystalline layers of long chain compounds, and it is evident that the barium phthioate in the multilayer is present in crystalline or at least smectic form.

In the crystalline state, long chain compounds may be arranged in vertical or tilted single molecule or double molecule lattices (19). Barium salts of straight chain fatty acids crystallize in the vertical double molecule arrangement. The only branched chain acids that it has so far been possible to obtain in multilayer form, namely  $\alpha$ -methyl-substituted long chain acids (calcium salts (20)), show a tilted double molecule arrangement. The structure of phthioic acid is unknown, but it can be shown that the spacing of 35 Å. for barium phthioate must correspond to a vertical or slightly tilted *double* molecule arrangement.

No measurements of the long spacing of the barium salt of the normal isomer of phthioic acid, *n*-hexacosanoic acid, have been found in the literature, but an extrapolation from the spacings given by the lower homologues shows that the spacing of barium hexacosanoate will be about 70 Å., corresponding to about 35 Å. per molecule. A single layer arrangement of barium phthioate can be excluded, as the straight chain isomer even in a vertical single layer arrangement would not give a spacing longer than 35 Å. and the height of a single layer of the branched phthioic acid molecule must necessarily be lower. This, together with the fact that salts of carboxylic acids have never been found to form single layer lattices, indicates that the spacing of 35 Å. corresponds to a double molecule arrangement. The possibility that the first line observed in the x-ray pattern is the second order and that all odd orders are absent can also be excluded. If the odd orders were

absent, the true spacing would be  $2 \times 35 \text{ \AA.} = 70 \text{ \AA.}$  and this is just the spacing that can be obtained with two vertical straight chain molecules with 26 carbon atoms in the chain and could not possibly be given by a double molecule arrangement of a branched chain acid. Furthermore, in the diffraction patterns given by fatty acids and their salts, odd orders of the long spacing are always stronger than the even orders.

The spacing of  $35 \text{ \AA.}$  observed for the barium salt of the phthioic acid, although only about half that of the isomeric straight chain acid, must therefore correspond to 2 vertical or slightly tilted molecules, giving a spacing *per molecule* of  $17.5 \text{ \AA.}$

It is of course not certain that the phthioate multilayer is truly crystalline; it may also be a smectic arrangement within the film. Barium salts of straight chain acids are not smectic, however, (21) and the above discussion will apply in either case.

The equimolecular mixed phthioic acid-octadecylamine film gave a weak but sharp diffraction pattern with a long spacing of  $31.3 \text{ \AA.}$  The interpretation of this pattern is difficult, however. A film of pure octadecylamine (octadecylamine phosphate) built from the same substrate as the mixed film gave a long spacing of about  $48 \text{ \AA.}$  It might be possible to obtain more information on the mixed multilayers by using different molecular proportions of phthioic acid and octadecylamine and amines of different chain lengths. If the spacing of  $31.3 \text{ \AA.}$  remains constant, it must be due to the phthioic acid. It has not yet been possible to carry out such experiments.

A powder photograph of the free acid taken at a temperature a few degrees below the melting point showed only a very diffuse ring corresponding to a spacing of  $4.2 \text{ \AA.}$ , which is the side spacing given by a rotating hydrocarbon chain (22).

#### DISCUSSION

##### *Structure Suggested by Film Experiments*

The outstanding film properties of phthioic acid are that (a) at low temperatures the acid, although highly branched, forms condensed monolayers which cannot be compressed below  $36 \text{ sq. \AA.}$  per molecule without collapse, (b) the un-ionized acid has an exceptionally high apparent surface moment, (c) from a suitable substrate containing barium ions multilayers of barium phthioate

are easily obtained. Phthioic acid is the only branched acid so far tried from which perfect multilayers have been obtained. (d) Multilayers of barium phthioate give good x-ray diffraction patterns showing a long ( $c$ -) spacing of 35 Å.

The fact that the lowest stable area of the mesomorphous film is  $36 \pm 1$  sq. Å. and the very short  $c$ -spacing of the barium phthioate would suggest that a molecule mainly consists of two parallel hydrocarbon chains forming some sort of "hairpin" molecule. An area of 18 sq. Å. per chain might appear to be too small, as the cross-section of a hydrocarbon chain in the crystalline state is 18.1 to 18.5 sq. Å. and the smallest areas per chain obtained in monolayers are usually slightly larger (23). The larger monolayer cross-section may be due to the head group and its attached water molecules. Under suitable experimental conditions, however, areas of 18.5 sq. Å. per chain can be obtained in monolayers of straight chain compounds (24, 25). In the case of phthioic acid there is only one head group, and at an area of 36 sq. Å. there is ample room for the carboxyl group and the full pressure is applied to the hydrocarbon part of the molecule. 13 dynes per cm. on a monolayer 18 Å. high are equal to 73.6 kilos per sq. cm. Some of the "hairpin" molecules studied in Paper II (7) could be compressed down to 37 sq. Å. per molecule.

#### *Comparison with Some Branched Acids of Known Constitution*

The results obtained with the  $C_{26}$  isomers of phthioic acid prepared by Chargaff (26) and studied in Paper I (6) show that two long hydrocarbon chains attached to the same tertiary carbon atom do not pack side by side easily; for close side by side packing to occur it is necessary, as shown in Paper II (7), to replace the hydrogen atom of the tertiary carbon atom by an alkyl group. The simplest structure that would seem to fit these facts is that of a trisubstituted acetic acid with two long chains. This structure also accounts for the very high surface moment of the phthioic acid, which is very much higher than those of the disubstituted acetic acid isomers but about equal to that given by methyl-di-*n*-octylacetic acid.

A comparison between phthioic acid and some branched acids of known structure is shown in Figs. 6 and 7. Curve *a*, Fig. 6, shows methyl-di-*n*-octylacetic acid. The force-area and surface

moment-area curves of this trisubstituted acetic acid resemble rather closely those of phthioic acid. Curve *b*, Fig. 6, shows di-*n*-octylacetic acid, which is more expanded and has a lower moment than the corresponding trisubstituted acetic acid. *n*-Decyl-*n*-tetradecylacetic acid, one of the disubstituted acetic acid isomers of the phthioic acid prepared by Chargaff (26), gives the results shown in Curve *c*, Fig. 6. It is more expanded and has a

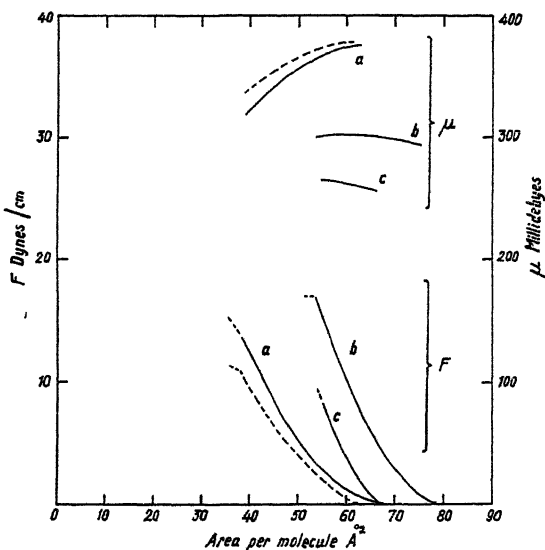


FIG. 6. Comparison between phthioic acid (dotted curves) and some branched acids of known constitution. Curve *a*, methyl-di-*n*-octylacetic acid ( $\text{C}_{19}\text{H}_{38}\text{O}_2$ ); Curve *b*, di-*n*-octylacetic acid ( $\text{C}_{18}\text{H}_{36}\text{O}_2$ ); Curve *c*, *n*-decyl-*n*-tetradecylacetic acid ( $\text{C}_{26}\text{H}_{52}\text{O}_2$ ). 0.01 *N* HCl substrate at 20°.

considerably lower moment than phthioic acid. Of the two other isomers prepared by Chargaff, namely di-*n*-dodecylacetic acid and *n*-butyl-*n*-eicosylacetic acid, the first is still more expanded than the *n*-decyl-*n*-tetradecylacetic acid and the second forms no stable monolayer on acid substrate at room temperature (6).

The result shown in Fig. 7, Curve *a*, for dimethyl-*n*-decylacetic acid<sup>2</sup> indicates that a trisubstituted acetic acid with two short and

<sup>2</sup> Stållberg and Stenhagen, unpublished measurements.

one long chain does not possess a very high surface moment. Curve *b* shows phytanic acid, 3,7,11,15-tetramethylhexadecic acid. This structure is of interest, as Spielman and Anderson (5) have suggested that phthioic acid is a long chain structure with several side chains. Owing to the presence of four evenly distributed methyl side chains, phytanic acid forms a rather ex-

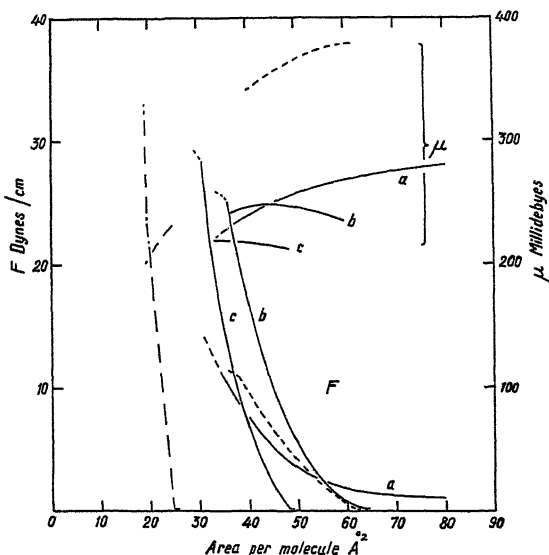


FIG. 7. Comparison between phthioic acid (dotted curves) and some branched acids of known constitution. Curve *a*, dimethyl-*n*-decylacetic acid ( $C_{14}H_{28}O_2$ ); Curve *b*, 3,7,11,15-tetramethylhexadecic acid ( $C_{20}H_{40}O_2$ ); Curve *c*, tuberculostearic acid (10-methylstearic acid,  $C_{19}H_{38}O_2$ ). The dash-dotted curves to the left, the normal chain margaric acid ( $C_{17}H_{34}O_2$ ). 0.01 N HCl substrate at 20°.

panded film. The surface moment of the acid, which has the first methyl side chain in  $\beta$  position, is not appreciably higher than that of the normal chain isomer and on a substrate containing barium ions<sup>1</sup> the monolayer can be compressed down to an area of 30 sq. Å. per molecule before collapse occurs. As an increase in the total number of carbon atoms in a structure of this type would probably result in a still smaller minimum area, it appears

that if the phthioic acid is a long chain structure with several side chains at least some of these must be longer than methyl. The x-ray results do not appear to be easily compatible with a structure of this type, however. Curve *c*, Fig. 7, shows tuberculostearic acid (10-methylstearic acid).<sup>2</sup> This acid has 19 carbon atoms as against 20 in the phytanic acid, but the presence of only one methyl side chain makes the monolayer less expanded than that of the latter. The side chain is very far away from the carboxyl group and the apparent surface moment of the 10-methylstearic acid is not appreciably different from that of normal chain acids (*cf.* margaric acid in Fig. 7).

It should be pointed out that in the above discussion acids of different molecular weight have been compared. It is known that the surface moment changes slightly with increase in molecular weight in normal chain and also in symmetrical branched acids, such as dialkylacetic acids (6). The changes due to increase in molecular weight are much smaller, however, than those here discussed, which depend on the special configuration of the molecule near the carboxyl group.

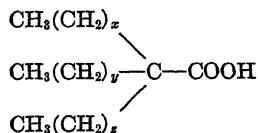
The only branched aliphatic acids so far investigated which have moments as high as phthioic acid are methyldi-*n*-octylacetic acid and 3-methyl-3-octylundecic acid (methyldi-*n*-octyl-( $\beta$ )-propionic acid) (7).

The deposition ratio for the multilayers of barium phthioate is very nearly equal to 1, and the optical spacing per layer,  $18.2 \pm 0.7 \text{ \AA.}$ , will correspond closely to the height of the monolayer on water (12). If phthioic acid mainly consists of two parallel chains, these must at an area of  $36.5 \text{ sq. \AA.}$  be closely packed and vertical and the optical spacing of the multilayer is then nearly equal to the length of the molecule. It is interesting that the x-ray spacing per molecule of the barium salt, in which the molecules are vertical or slightly tilted (and the spacing per molecule therefore is equal to or slightly shorter than the length of the molecule),  $17.5 \text{ \AA.}$ , is almost identical with the optical spacing of the multilayer. The dimensions of the phthioic acid molecule are therefore approximately  $18 \text{ \AA.} \times 36.5 \text{ sq. \AA.}$

Phthioic acid is optically active and must contain at least 1 asymmetric carbon atom. To account for this, the long chains



in our structure must be of unequal lengths. The suggested structure is perhaps best expressed as follows:



where  $x$  and  $y = 8\text{--}13$  and are different,  $z = 0$  or  $1$ ;  $x + y + z = 21$ .

*Trisubstituted Acetic Acid Structure and Other Properties  
of Phthioic Acid*

It now remains to investigate whether the trisubstituted acetic acid structure can account for all other known properties of phthioic acid.

Chargaff (26) concluded from the melting points of the disubstituted acetic acid isomers of phthioic acid that the latter must contain at least three hydrocarbon chains. By the Kuhn-Roth method of estimating side chain methyl, Wagner-Jauregg (27) found that tuberculostearic acid gave 1.4 molecules of acetic acid and phthioic acid 2.4 molecules of acetic acid per molecule. The phthioic acid used by Wagner-Jauregg was probably inhomogeneous, but the results suggest the existence of at least three carbon chains.

Spielman and Anderson (5) carried out experiments with a view to determining whether phthioic acid is substituted in the  $\alpha$  position. From their results phthioic acid is not a primary acid but it does not seem possible to decide whether it is a secondary or tertiary acid. Wieland degradation yielded mainly a mixture of neutral substances. A primary acid would give an acid and a secondary acid, a ketone. Spielman and Anderson concluded that the results were in favor of  $\alpha$  substitution. Determination of the chlorine number by the method of von Braun gave ambiguous results. The theoretical chlorine numbers for primary and secondary acids are 200 and 100 respectively and a tertiary acid should give no reaction. For phthioic acid, chlorine numbers of 140, 220, and 480 were obtained and it was concluded that in the case of phthioic acid the method could not be relied upon as a criterion for  $\alpha$  substitution.

Chromic acid oxidation of phthioic acid, also carried out by

Spielman and Anderson (5), gave an acid  $C_{11}H_{22}O_2$ , the 2,4,6-tribromoanilide of which melted  $18^\circ$  and the *p*-bromophenacyl ester  $19-20^\circ$  lower than the corresponding derivatives of *n*-undecic acid. The acid, therefore, is not *n*-undecic acid, and Spielman and Anderson suggested that it had a branched chain. It appears quite possible, however, that the  $C_{11}$  acid obtained by Spielman and Anderson is a mixture of normal chain acids of a mean molecular weight corresponding to  $C_{11}H_{22}O_2$ . The trisubstituted acetic acid structure on oxidation with chromic acid might be expected to give a mixture of normal chain acids, among which the  $C_{x+1}$  and the  $C_{y+1}$  acids should be found.

An x-ray investigation of the  $C_{11}$  acid obtained from phthioic acid might give valuable information on its composition.

Spielman and Anderson found no trace of dibasic acids or of neutral material among the oxidation products of phthioic acid. A long chain structure with a number of side chains might be expected to give dibasic acids on oxidation. From the oxidation (chromic acid) products of tuberculostearic acid (10-methylstearic acid) Spielman (28) was able to isolate azelaic acid and methyl-*n*-octyl ketone. On the other hand, a trisubstituted long chain acetic acid cannot easily be expected to give any dibasic acids on oxidation with chromic acid.

One difficulty for the trisubstituted acetic acid structure might arise from the fact that phthioic acid appears to be relatively easily esterified and the esterification rate of  $\alpha, \alpha$ -substituted long chain acids might be expected to be low (29). Experiments on some trisubstituted long chain acetic acids prepared by Dr. A. J. Birch in the laboratory of Professor Sir Robert Robinson, show, however, that the esterification, although considerably slower than for normal acids, is not unduly difficult.<sup>3</sup>

Levene, Rothen, and Marker (30) have examined the optical properties of a number of fatty acids with methyl side chains and found that the molecular rotation diminished when the methyl group was displaced successively further away from the carboxyl group. As pointed out by Spielman and Anderson (5), the relatively high molecular rotation of phthioic acid,  $+50^\circ$ , thus indicates an asymmetric carbon atom in  $\alpha$ ,  $\beta$ , or  $\gamma$  position, preferably in  $\alpha$  position.

<sup>3</sup> Personal communication from Dr. Birch.

The melting point of methyl-di-*n*-octylacetic acid, prepared by Birch,<sup>3</sup> is about  $-10^{\circ}$  and the melting point of phthioic acid,  $+20$  to  $21^{\circ}$ , is about that expected for a higher homologue containing 7 carbon atoms more.

The trisubstituted acetic acid structure thus appears to be able to account for all known properties of phthioic acid in a fairly satisfactory manner. The correctness of this structure and the finer details can of course only be determined by ordinary methods of organic chemistry, when larger quantities of the acid are available.

As acids of the structure here proposed for the phthioic acid represent a hitherto unknown type of fatty acids, it was originally intended to carry out experiments with a synthetic acid of this type, isomeric with phthioic acid, before publication of these results. Professor Sir Robert Robinson kindly agreed to undertake to prepare ethyl-*n*-decyl-*n*-dodecylacetic acid (1), and Dr. A. J. Birch,<sup>4</sup> working in Professor Robinson's laboratory, had nearly completed this synthesis when the European conditions made communications with the Oxford laboratory impossible.

Part of this work was carried out in 1938 by one of us (E. S.), during the tenure of a Rockefeller Foundation fellowship at the Department of Colloid Science, Cambridge, England. He is indebted to Professor E. K. Rideal for his interest in the work and to Dr. I. Fankuchen for help with part of the x-ray work.

We are greatly indebted to Professor R. J. Anderson for a specimen of phthioic acid that made this work possible, and for his continued interest in the work.

The work at Upsala has been aided by grants from the Swedish Association against Tuberculosis and from the Rockefeller Foundation.

<sup>4</sup> In collaboration with Dr. Årén and Dr. T. Teorell at Upsala some branched acids prepared by Dr. Birch in connection with this work, namely dimethyl-*n*-dodecylacetic acid, methyl-di-*n*-octylacetic acid, and 3-methyl-3-octyl-*n*-undecic acid, have been subjected to a preliminary biological test on guinea pigs. The results indicate that the trisubstituted acetic acids mentioned above are rather toxic, but no specific tissue reaction has been obtained so far. On the other hand the ( $\beta$ )-trisubstituted propionic acid (3-methyl-3-octyl-*n*-undecic acid) is apparently hardly toxic at all. This difference between  $\alpha$ - and  $\beta$ -trisubstituted acids must await confirmation when more synthetic material is available, however.

## SUMMARY

Mono- and multimolecular films of phthioic acid have been studied.

Phthioic acid forms at low temperatures mesomorphous monolayers, which cannot be compressed below  $36 \pm 1$  sq. Å. without collapse.

The apparent surface moment of the undissociated acid, 380 to 350 millidebyes, is the highest value so far recorded for a saturated aliphatic acid. From a suitable substrate containing barium ions, Langmuir-Blodgett multilayers of barium phthioate are easily obtained. Such multilayers, built from a monolayer on water compressed to an area of 36 sq. Å. per molecule, show an optical spacing per layer of  $18.2 \pm 0.5$  Å. The barium phthioate multilayer gives a good x-ray diffraction pattern showing a long spacing of  $35 \pm 0.7$  Å.

The film measurements and a comparison with the results obtained with branched acids of known constitution suggest that the phthioic acid may be trisubstituted acetic acid with two long and one short chain. The correctness of this view can only be determined by further work, but the suggested structure appears compatible with all known properties of phthioic acid.

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## HEAVY CARBON AS A TRACER IN HETEROTROPHIC CARBON DIOXIDE ASSIMILATION\*

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The first proof that heterotrophic, non-photosynthetic bacteria can fix carbon dioxide was presented in 1935 by Wood and Werkman (15). This investigation introduced the first experimental evidence that carbon dioxide serves as a building material in synthetic reactions of all forms of life, whether simple or complex. The synthesis of citric acid from oxalacetate by avian tissue was shown by Krebs and Johnson in 1937 (8), and Wood and Werkman (17) suggested that this reaction involved utilization of carbon dioxide. The recent results of Evans and Slotin (7) with radioactive carbon ( $C^{14}$ ) provide the experimental evidence showing that this is the case. The California group (Barker *et al.* (1, 2); Ruben and Kamen (14); Carson and Ruben (6); Carson *et al.* (5)), using  $C^{14}$ , has confirmed and extended our knowledge of heterotrophic carbon dioxide assimilation. They demonstrated fixation of carbon dioxide with yeast and a number of heterotrophic bacteria.

The present report concerns the mechanism of carbon dioxide fixation. Heavy carbon ( $C^{13}$ ) has been used as a tracer of fixed carbon dioxide. Such studies undoubtedly will lead to a clearer understanding of the chemistry of photosynthesis and chemosynthesis in general. The present results show the distribution of fixed carbon dioxide in the products of bacterial fermentation (19).

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## EXPERIMENTAL

The procedure consisted in fermenting a substrate with a cell suspension in a medium containing  $\text{NaHCO}_3$  enriched with  $\text{C}^{13}$ . The products were fractionated, determined quantitatively, converted to carbon dioxide, and the  $\text{C}^{13}$  content determined with the mass spectrometer (Nier (9)).<sup>1</sup> The sodium bicarbonate was prepared from carbon dioxide derived from methane, which contained a high complement of  $\text{C}^{13}$  and was obtained with the thermal diffusion column described by Nier (10). The methods of fractionation and quantitative determination of products were those previously employed. The alcohol in the neutral distillate, volatile acids in the steam distillate, and silver succinate from the ether extract were oxidized to carbon dioxide with persulfate (Osburn and Werkman (12)). When formic acid was present, it was removed from the volatile acid distillate by oxidation to carbon dioxide with mercuric oxide (Osburn *et al.* (13)) prior to the persulfate oxidation. Lactic acid and the non-reducing material were not analyzed for  $\text{C}^{13}$ .

The carbon recovery is expressed in per cent recovery of the carbon in the original substrate plus that in the sodium bicarbonate. Compounds in which the  $\text{C}^{13}$  was not determined are assumed to contain the natural complement of  $\text{C}^{13}$  in these calculations. The content of  $\text{C}^{13}$  is expressed as per cent  $\text{C}^{13}$ ; *i.e.*,

$$\frac{\text{Moles } \text{C}^{13}}{\text{Moles } \text{C}^{12} + \text{moles } \text{C}^{13}} \times 100$$

It should be noted that the per cent  $\text{C}^{13}$  is an expression of the quantitative relationship of  $\text{C}^{12}$  and  $\text{C}^{13}$  in the compound and is not an expression of the actual amount of  $\text{C}^{13}$ . The amount of  $\text{C}^{13}$  present in a compound is calculated as follows: per cent  $\text{C}^{13} \times \text{mm of compound} \times \text{number of carbon atoms in compound} = \text{mm of } \text{C}^{13}$ . The fact that a compound contains fixed carbon dioxide is indicated when the content of  $\text{C}^{13}$  is above that of the normal complement of  $\text{C}^{13}$ ; *i.e.*, 1.09 per cent. Nier and Gulbransen (11) have shown that all natural products examined contained approximately 1.09 per cent  $\text{C}^{13}$ .

<sup>1</sup> We wish to thank Miss M. Svensen for assistance in the preparation of gas samples.

It should be emphasized that, in the comparison of the amount of fixation of carbon dioxide in one fermentation with that of another, both the concentration of  $C^{13}$  in the original sodium bicarbonate and the extent of dilution of this bicarbonate-carbon dioxide by carbon dioxide formed from the substrate must be considered.

TABLE I

*Distribution of Fixed Carbon among Products of Galactose and Pyruvate Fermentation by Escherichia coli*

Substrate.....	Galactose*		Pyruvate†	
$C^{13} + C^{12}$ recovery, per cent. . . . .	96.9		98.7	
$C^{13}$ recovery, per cent. . . . .	94.3		101.2	
	mm per l.	per cent $C^{13}$	mm per l.	per cent $C^{13}$
Initial substrate.....	75.0	1.09	175.0	1.09
Final substrate.....	7.3		30.0	
Initial $NaCHO_3$ .....	110.0	4.17	149.1	2.58
Final $NaHCO_3$ and $CO_2$ ..	143.8	2.72	167.8	2.80
Products				
Ethyl alcohol.....	51.4	1.06	0.0	
Acetic acid.....	20.6	1.07	87.2	1.07
$CO_2$ .....	33.8		18.7	
Formic acid.....	1.12		70.2	1.50
Succinic acid.....	52.5	1.46	34.3	1.24
Lactic acid.....	0.0		8.6	
$H_2$ .....	12.0		31.1	

\* The reaction mixture = 0.075 M galactose, 0.109 M  $NaHCO_3$ , and 2.0 per cent wet bacteria (*Escherichia coli* 26 from 20 hour culture at 30° in glucose 1.0 per cent, peptone 0.3 per cent,  $(NH_4)_2SO_4$  0.3 per cent,  $K_2HPO_4$  1.0 per cent, and tap water 10 per cent). In this and subsequent experiments of Tables I, II, and III the reaction time was 3 days, temperature 30°, atmosphere  $N_2$ .

† The reaction mixture = 0.175 M sodium pyruvate, 0.15 M  $NaHCO_3$ , and 2.0 per cent wet bacteria (*Escherichia coli* 26 from 15 hour culture at 37° on Lemco beef extract 1 per cent, Difco peptone 1 per cent, Difco yeast extract 0.5 per cent, NaCl 0.5 per cent, agar 3.0 per cent).

On consideration of the fermentation of galactose by *Escherichia coli* (Table I), it is apparent that succinic acid is the only product which contained  $C^{13}$  in greater than normal concentration and that the excess of  $C^{13}$  must have originated by carbon dioxide fixation from the initial sodium bicarbonate which contained 4.17 per cent



C<sup>13</sup>. The usual limit of error in the determination of C<sup>13</sup> is about 2 per cent. The value 1.46 for succinic acid is, therefore, highly significant. There is reason to believe that succinic acid is formed by union of a 3-carbon compound originating from the galactose and a 1-carbon compound, carbon dioxide, originating from both the sodium bicarbonate and galactose (18, 20). On this basis 3 carbon atoms of the succinic acid should have the natural complement of C<sup>13</sup> (1.09 per cent), and the 4th carbon atom an unknown amount, depending on the concentration of C<sup>13</sup> in the initial sodium bicarbonate, and dilution by carbon dioxide originating from the galactose. On the basis of these requirements, the greatest possible C<sup>13</sup> concentration that can be obtained in the succinic acid will be 1.86 per cent;<sup>2</sup> i.e., when all the fixed carbon dioxide is from the original sodium bicarbonate containing 4.17 per cent C<sup>13</sup>. Calculated on the basis of the fixed carbon dioxide containing 2.72 per cent C<sup>13</sup>, the amount present in the sodium bicarbonate and carbon dioxide at the conclusion of the fermentation, the percentage of C<sup>13</sup> in the succinate is 1.50 compared to the observed value of 1.46.

The quantitative relationship of the end-products is in accord with the indicated carbon dioxide fixation. Most schemes of fermentation postulate that the formation of a 2-carbon compound is accompanied by a 1-carbon compound. In the galactose fermentation 72 mm of 2-carbon compounds were formed and only 35 of 1-carbon compounds. The deficiency in 1-carbon compounds is undoubtedly caused by carbon dioxide assimilation.

The fermentation of pyruvate by *Escherichia coli* (Table I) is also accompanied by fixation of carbon dioxide in succinic acid.<sup>3</sup>

<sup>2</sup> Calculated by the following general equation,  $(1.09 N + X)/M =$  average per cent C<sup>13</sup> in molecule. 1.09 = per cent C<sup>13</sup> in normal carbon,  $N$  = No. of carbon atoms with normal complements of C<sup>13</sup>,  $X$  = per cent C<sup>13</sup> in-fixed carbon dioxide, and  $M$  = total No. of carbon atoms in molecule.

<sup>3</sup> Considerable difficulty was encountered in obtaining a substantial yield of succinate from pyruvate. Two factors are particularly important, (1) the method employed in growing the cells, and (2) the concentration of sodium bicarbonate in the reaction mixture. Cells grown in glucose broth or on glucose-peptone-agar gave low yields of succinic acid, whereas cells grown on beef extract-yeast extract-peptone-agar gave high yields. With the latter cells the yield of succinic acid per 100 mm of fermented pyruvic acid was 4.3, 10.0, 16.8, and 19.6 mm with 0.025, 0.050, 0.100, and 0.175 M sodium bicarbonate respectively, under an atmosphere of carbon dioxide.

The  $C^{13}$  concentration in this acid was 1.24 per cent. The  $C^{13}$  value of formic acid, 150 per cent, indicates that a substantial amount of carbon dioxide was fixed in this acid. These results confirm Woods' (22) experiments in which carbon dioxide was reduced to formic acid with hydrogen by *Escherichia coli*. In comparison of the succinic acid and formic acid values, it must be borne in mind that three of the carbon atoms of succinic acid may contain the natural complement of  $C^{13}$  and that these carbon atoms dilute the  $C^{13}$  of the fixed carbon.

According to the usual schemes of pyruvate fermentation an initial equivalence of 2- and 1-carbon compounds is to be expected from the 3-carbon compound. This equality will be destroyed if one of these compounds is consumed at a greater rate than the other. It is interesting, therefore, that in spite of carbon dioxide assimilation in the fermentation of pyruvate, the sum of the 1-carbon compounds (88.9 mm) was equal to the 2-carbon compound (87.2 mm). This equality has also been observed in a number of unpublished experiments. The question thus arises as to whether the 2-carbon and 1-carbon compounds were initially formed in equal molar quantities, and if they were, why the 2-carbon compound was broken down in an amount equivalent to the fixed carbon dioxide.

Results of the fermentation of glycerol by the propionic acid bacteria are shown in Table II. Two fermentations (Nos. 1 and 2), identical except that Fermentation 2 contained sodium bicarbonate with concentrated  $C^{13}$ , 4.64 per cent, were conducted for comparison. A survey of the results shows that carbon dioxide was fixed in the succinic acid, volatile acids, and propyl alcohol and that the distribution of  $C^{13}$  was normal in all products when the natural sodium bicarbonate was used. Succinic acid contained the greatest percentage of fixed carbon, 1.65 per cent  $C^{13}$  as compared to 1.48 per cent for the volatile acids. However, since approximately 3 times as much volatile acid as succinic acid is produced in the fermentation, more of the total fixed carbon is in the volatile acids. It is somewhat difficult to estimate the exact amount of fixed carbon in a particular compound and the percentage this carbon constitutes of the total fixed carbon. The difference between the mm of  $C^{13}$  in the original sodium bicarbonate (7.49) and that in the final sodium bicarbonate and carbon dioxide

(4.54) is not a measure of fixed carbon dioxide. This fact is apparent when one considers that the final sodium bicarbonate and carbon dioxide contain not only the  $C^{13}$  of the residue of the original sodium bicarbonate but also  $C^{13}$  of the carbon dioxide formed from the substrate which contains the natural complement of  $C^{13}$ . Since the products in the two fermentations (Nos. 1

TABLE II

*Distribution of Fixed Carbon among Products of Glycerol Fermentation by Propionibacterium pentosaceum*

Fermentation No. ....	1			2			3	
$C^{13} + C^{12}$ recovery, per cent	104.5			104.4			97.5	
$C^{13}$ recovery, per cent ....	104.4			100.5			95.2	
	mm per l.	per cent $C^{13}$	mm $C^{13}$ per l.	mm per l.	per cent $C^{13}$	mm $C^{13}$ per l.	mm per l.	per cent $C^{13}$
Initial glycerol.....	175.0	1.09	5.72	175.0	1.09	5.72	125.0	1.09
Final glycerol.....	2.5		0.08	2.0		0.06	0.9	
Initial $NaHCO_3$ (a)...	161.5	1.10	1.78	161.4	4.64	7.49	108.1	2.56
Final $NaHCO_3$ and $CO_2$ (b).....	123.6	1.09	1.35	125.3	3.62	4.54	80.1	2.10
Apparent $CO_2$ fixed (a - b).....	37.9			36.1			28.0	
Products								
Succinic acid.....	36.4	1.10	1.60	37.0	1.65	2.44	21.1	1.28
Propionic " .....	114.4	1.09	3.86	114.5	1.48	5.27	90.6	1.23
Acetic acid.....	5.4			6.3			6.1	
Propyl alcohol....	9.5	1.08	0.31	9.0	1.39	0.38	6.7	1.18
Non-reducing material.....	9.7		0.63	9.0		0.59	0.0	

The reaction mixture = 0.175 M glycerol, 0.175 M  $NaHCO_3$ , and 2.0 per cent wet bacteria (*Propionibacterium pentosaceum* 49W grown 6 days at 30° on 0.5 per cent glycerol, 0.05 M phosphate buffer, pH 7.0, and 0.4 per cent Difco yeast extract).

and 2) are quantitatively practically identical, an approximation of the fixed carbon dioxide can be obtained by comparing the fermentation containing normal sodium bicarbonate with that containing  $C^{13}$ -enriched sodium bicarbonate. The mm of  $C^{13}$  in each product of the fermentation with concentrated  $C^{13}$ , in excess of the mm of  $C^{13}$  in the products of the normal fermentation, are a measure of the fixed carbon dioxide. The excess  $C^{13}$  in the succinic acid is 0.84 mm, in the volatile acids 1.41 mm, and in propyl

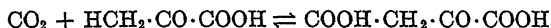
alcohol 0.07 mm. Apparently, about 36 per cent of the fixed carbon dioxide is in the succinic acid and 61 per cent in the volatile acid. Carson and Ruben (6) obtained similar results with  $C^{11}$ .

It should be noted that notwithstanding the net decrease in total carbon dioxide (sodium bicarbonate and gaseous carbon dioxide) during the fermentation, there was a production of carbon dioxide from the glycerol. This fact is evident since the per cent  $C^{13}$  of the final sodium bicarbonate plus carbon dioxide is less than that of the original sodium bicarbonate (*cf.* Fermentations 2 and 3). The  $C^{13}O_2$  has been diluted with  $C^{12}O_2$  formed from the glycerol. The production of carbon dioxide equivalent to the acetic acid is not sufficient to account for the observed dilution of  $C^{13}$ . It is thus probable that there is considerable additional cleavage of the glycerol. The fermentation therefore may involve more complex conversions than a direct formation of propionic acid from the glycerol and synthesis of succinic acid through the union of a 3-carbon compound from the glycerol and a 1-carbon compound from the sodium bicarbonate. The total carbon dioxide fixed in the fermentation of glycerol is, therefore, greater than the apparent fixed carbon dioxide. The apparent fixed carbon dioxide is the decrease in total carbon dioxide of the system, *i.e.* (original sodium bicarbonate) minus (final sodium bicarbonate + carbon dioxide), and therefore does not include fixed carbon dioxide originating from the glycerol.

The question arises as to whether or not the  $C^{13}O_2$  may have been diluted by  $C^{12}O_2$  from the glycerol owing to an exchange reaction. While it is improbable that non-enzymic exchange occurs, there may be interchange of  $CO_2$  during the enzymic conversions. For example, if pyruvic acid is formed intermediately from glycerol during the formation of propionic acid, it is conceivable that in the presence of carboxylase there would be exchange of  $C^{13}O_2$  with the carboxyl carbon of the pyruvic acid.<sup>4</sup> The exchange reaction is, of course, in the final analysis, fixation of carbon dioxide. It will have no qualitative influence on the products of fermentation, however, unless it results in the forma-

<sup>4</sup> Proof that the fixed carbon is exclusively in the carboxyl group of succinic and propionic acids is given by Wood *et al.* (20, 21). Our results are not in agreement with the report of Carson *et al.* (5) in which it is suggested that the fixed carbon is in all three carbon groups of propionic acid.

tion of an intermediate which would not otherwise be formed from the substrate. Also, the exchange can cause a net uptake of carbon dioxide (such as occurs in the glycerol fermentation) only when it involves a synthesis with an intermediate which is formed from the substrate without evolution of carbon dioxide. The cleavage of glucose and many other substrates is undoubtedly to a 3-carbon compound and ultimately to pyruvic acid without simultaneous formation of carbon dioxide. The following equilibrium,



involves an exchange. If, however, the oxalacetic acid is removed by reduction, a plausible explanation is offered for the formation of 4-carbon dicarboxylic acids containing fixed carbon, as well as for the observed net uptake of carbon dioxide.

The experimental values are in fairly good agreement with this supposition. Calculated on the basis that the fixed carbon dioxide has a concentration of  $\text{C}^{13}$  equal to that at the conclusion of the fermentation<sup>5</sup> and that the  $\text{C}^{13}\text{O}_2$  is fixed in one carboxyl group of succinic acid, the values, 1.72 (Fermentation 2) and 1.34 (Fermentation 3), are obtained for the per cent  $\text{C}^{13}$  in succinic acid as compared to the experimental values 1.65 and 1.28 per cent respectively. Calculated on the basis that two carboxyl groups contain fixed carbon (*i.e.*, carbon dioxide exchange in both carboxyl groups) the values are 2.71 per cent (Fermentation 2) and 1.77 per cent (Fermentation 3). The data, therefore, indicate that carbon dioxide is fixed in only one carboxyl group of succinic acid.

The mechanism by which carbon is fixed in the carboxyl group of propionic acid is more uncertain. A possible explanation is that decarboxylation of succinic acid occurs to yield propionic acid and carbon dioxide (*cf.* Wood and Werkman (16)). This decarboxylation would yield propionic acid with  $\text{C}^{13}$  in the carboxyl group and also dilute the  $\text{C}^{13}\text{O}_2$ , since from a symmetrical dicarboxylic acid containing fixed carbon in one carboxyl group, there

<sup>5</sup> The concentration of  $\text{C}^{13}$  in the fixed carbon dioxide is difficult to estimate. There is no assurance that the carbon dioxide produced within the cell comes completely to equilibrium with the dissolved carbon dioxide in the medium before it is utilized. Arbitrarily in this and subsequent experiments we have used the concentration of  $\text{C}^{13}$  in the carbon dioxide at the conclusion of the fermentation as representative of the average concentration of  $\text{C}^{13}\text{O}_2$  available to the cell.

is an equal chance of splitting off carbon dioxide from the carboxyl which originated from the glycerol, or  $C^{13}O_2$  from the carboxyl formed from the fixed carbon. A requirement of this reaction is that the concentration of heavy carbon in the carboxyl group of propionic acid be no greater than the average concentration of the  $C^{13}$  in the two carboxyl groups of succinic acid. It is interesting that the  $C^{13}$  in the carboxyl groups of succinic and propionic acids is practically equal<sup>6</sup> (Fermentation 2, 2.21 and 2.29 per cent; Fermentation 3, 1.47 and 1.54 per cent). The implication of this equality is that all the propionic acid may be formed via decarboxylation of succinic acid or some other symmetrical dicarboxylic acid and none by conversion of the intact glycerol to propionic acid.

The dilution of the  $C^{13}$  of the original  $NaHCO_3$  as calculated on the basis of the above scheme is in fair agreement with the observed values. Carbon dioxide containing the natural complement of  $C^{13}$  and equivalent to one-half of the quantity of propionic acid would be formed. Assuming the  $C^{13}$  of the original  $NaHCO_3$  is diluted by this amount of carbon dioxide, one obtains 3.76 per cent (Fermentation 2) and 2.23 per cent (Fermentation 3) for the concentration of  $C^{13}$  in the final carbon dioxide as compared to the observed values 3.62 and 2.10 per cent respectively. The somewhat lower observed values are to be expected because some carbon dioxide may be formed along with the acetic acid.

On the other hand the experimental values are not easily adaptable to an exchange reaction, for if the  $C^{13}O_2$  were fixed in the propionic acid by the exchange 3-carbon compound = 2-carbon compound + carbon dioxide and equilibrium was reached the  $C^{13}$  content would be greater in the propionic acid than is observed (1.93 per cent, Fermentation 2, and 1.42 per cent, Fermentation 3) (*cf.* foot-notes 2 and 5 for calculations) compared to experimental values 1.47 per cent, Fermentation 2, and 1.23 per cent, Fermentation 3. It is thus evident that if propionic acid is formed by reduction without splitting of the glycerol chain there is incomplete carbon dioxide exchange with the carboxyl carbon and by some coincidence the exchange was almost exactly 50 per cent in both fermentations, Nos. 2 and 3. It seems more probable that all

<sup>6</sup> These values have been calculated from the average per cent of  $C^{13}$  in the respective compounds as given in Table II and by means of the general equation of foot-note 2.

the propionic acid is formed by a mechanism involving union of a 3-carbon compound from the glycerol and carbon dioxide, yielding ultimately a symmetrical dicarboxylic acid (possibly succinic acid) and that this acid is subsequently converted to propionic acid and carbon dioxide. The liberated carbon dioxide is then used again in a similar cycle of events. The mechanism by which propionic acid is formed from lactic acid, glucose, etc., has always been in

TABLE III

*Distribution of Fixed Carbon among Products of Citrate and Glucose Fermentation by Citrobacter and Propionibacterium Respectively*

	Substrate fermented per liter	Succinic acid per 100 mM substrate	C <sup>13</sup> in succinic acid	C <sup>13</sup> in fermentation after removal of succinate and formate*	C <sup>13</sup> in original NaHCO <sub>3</sub>
	mM	mM	per cent	per cent	per cent
Citric acid, <i>Citrobacter intermedium</i> †.....	50.0	100.0	1.32	1.06	3.70
Glucose, <i>Propionibacterium pentosaceum</i> ‡....	100.0	41.5	1.38	1.30	4.18

\* The succinate was precipitated with AgNO<sub>3</sub> after removal of phosphate with Ba(OH)<sub>2</sub>. The formic acid was next oxidized with HgO. The residue was oxidized to CO<sub>2</sub> with persulfate and used in C<sup>13</sup> determination.

† The reaction mixture = 0.05 M sodium citrate, 0.125 M NaHCO<sub>3</sub>, 0.075 M phosphate buffer, pH 6.3, and 2.0 per cent wet bacteria (*Citrobacter* MSBK grown 4 days at 30° on 0.6 per cent citrate, 0.1 per cent peptone, 0.3 per cent (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05 per cent K<sub>2</sub>HPO<sub>4</sub>, 0.05 per cent MgSO<sub>4</sub> (pH 6.5)).

‡ The reaction mixture = 0.1 M glucose, 0.1 M NaHCO<sub>3</sub>, and 3.0 per cent wet bacteria, *Propionibacterium pentosaceum* 49W (cf. Table II for growth of bacteria).

doubt. In the past it has seemed probable that the propionic acid might be formed by removal of water from lactic acid to form acrylic acid which would be reduced to propionic acid. Up to the present time no one has been successful in demonstrating the reduction of acrylic acid by propionic acid bacteria. It now seems likely that propionic acid may not be formed in this way but, instead, through an unexpected mechanism of union of 3-carbon and 1-carbon compounds with subsequent cleavage to form the 3-carbon compound.

The results of two fermentations, in which only a partial fractionation of the products was made, are given in Table III. The succinic acid was isolated and analyzed for  $C^{13}$  and the remainder of the products, with formic acid removed, was analyzed *in toto*. The fermentation of citric acid by *Citrobacter* occurred with fixation of carbon dioxide only in the succinate (1.32 per cent  $C^{13}$ ) just as in the galactose fermentation by *Escherichia coli*. Brewer and Werkman (3, 4) have proposed a scheme of succinate formation from citrate by *Citrobacter* involving initial cleavage of citrate to oxalacetate and acetate with subsequent reduction of the oxalacetate to succinate. Apparently this scheme will have to be extended to include carbon dioxide fixation with formation of succinate.

The fact that 100 mm of succinate are formed from 100 mm of citrate under the condition of this experiment is of interest. This relationship has been observed in a number of unpublished experiments.

The fermentation of glucose by propionic acid bacteria was accompanied by fixation of  $C^{13}O_2$  in the succinate and other products just as in the fermentation of glycerol (Table III). These results offer the first direct proof that carbon dioxide is fixed in the fermentation of glucose, although Wood and Werkman (18) have previously offered indirect evidence.

#### SUMMARY

Wood and Werkman established carbon dioxide assimilation by heterotrophic bacteria in 1935; our knowledge of this general phenomenon has been extended in the present communication by the use of  $C^{13}$  as a tracer. Carbon dioxide fixed in the fermentation of galactose, pyruvic acid, and citric acid by *coli* bacteria, occurs solely in the succinic and formic acids; in the fermentation of glycerol and glucose by *Propionibacterium*, the fixed carbon dioxide is in the succinic acid, propionic acid, and propyl alcohol. The data obtained by determination of the fixed  $C^{13}$  are in agreement with the suggestion that succinic acid is formed by union of a 3-carbon compound and carbon dioxide and the propionic acid by decarboxylation of a symmetrical dicarboxylic acid containing fixed carbon dioxide in only one carboxyl group. According to



this concept all the carbon dioxide is fixed originally by union of 3-carbon and 1-carbon compounds.

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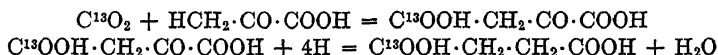
# THE POSITION OF CARBON DIOXIDE CARBON IN SUCCINIC ACID SYNTHESIZED BY HETERO- TROPHIC BACTERIA\*

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Wood and Werkman (9, 11) and Wood *et al.* (12) have presented evidence that succinic acid is formed in fermentations by heterotrophic bacteria through union of carbon dioxide and a 3-carbon compound formed from the substrate. They have suggested the following reactions for the synthesis,



A requirement of this scheme is that the fixed carbon be located exclusively in the carboxyl groups of the succinic acid. In the present report, through use of labeled carbon ( $\text{C}^{13}$ ) proof is provided that this is the location of the fixed carbon. These results, therefore, confirm the validity of the above reactions for succinic acid formation.

The 4-carbon dicarboxylic acids are key compounds in hydrogen transfer (7), the Krebs' cycle (3), and nitrogen fixation (8). An explanation of the mechanism of the formation of these acids is, therefore, of fundamental importance.

## EXPERIMENTAL

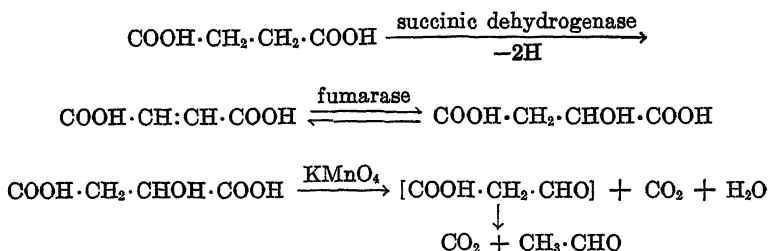
Two different methods have been used for the decarboxylation of biologically formed succinic acid containing  $\text{C}^{13}$  from fixed

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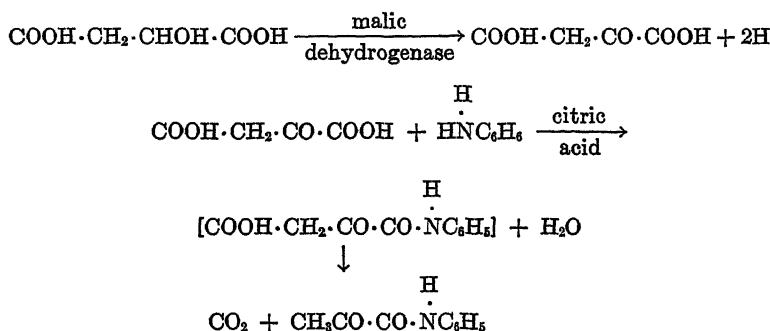
# 378 CO<sub>2</sub> Carbon in Succinic Acid Synthesis

C<sup>13</sup>O<sub>2</sub>. The first involves the enzymic conversion of the succinate to malate followed by permanganate oxidation to carbon dioxide and acetaldehyde.



It is apparent that 2 carbon dioxide molecules are formed from the carboxyl groups and acetaldehyde from the methylene groups of succinic acid. By determination of the C<sup>13</sup> content of the carbon dioxide and acetaldehyde the location of the fixed C<sup>13</sup> in succinic acid can be established. Actually, since an equilibrium is established by the fumarase, a mixture of fumarate and malate was oxidized. Fumarate, however, yields 2 molecules of carbon dioxide and only a trace of acetaldehyde. The carbon dioxide molecules originate from the carboxyl group and thus the presence of fumarate does not introduce an error.

The second method consists in the enzymic conversion of malate, formed as described above, to oxalacetate and decarboxylation with citric acid-aniline (Ostern (6)).



The carbon dioxide originates from one of the carboxyl groups of succinic acid. Determination of the C<sup>13</sup> in the carbon dioxide,

therefore, provides a measure of the fixed carbon in the carboxyl group.

The succinic dehydrogenase-fumarase mixture was prepared as described by Krebs (2). The biological succinate was from the fermentations described by Wood *et al.* (12) (Tables I and II). The succinic acid was extracted with ether after acid permanganate oxidation to remove lactate and other oxidizable compounds. The extract was steam-distilled to remove volatile acids. Enzymic conversion was carried out in 125 ml. flasks on a Warburg respirometer at 30°. The 30 ml. of reaction mixture contained 20 ml. of enzyme, 0.033 M succinate and 0.125 M phosphate buffer at pH 7.3. The mixture was incubated for 4.5 hours. It was then centrifuged and part was oxidized with permanganate (Friedemann and Kendall (1)) and the remainder was converted to oxalacetate.

The aldehyde from the permanganate oxidation was caught in a bisulfite tower which was connected to a tower containing one-half saturated potassium permanganate to absorb sulfur dioxide from the sulfite and this tower in turn was connected to an alkali tower to collect the carbon dioxide. The aldehyde was recovered by distilling a mixture of the bisulfite complex and calcium carbonate. Calcium carbonate breaks up the sulfite aldehyde addition compound. Dissolved carbon dioxide was removed from the aldehyde solution by precipitation as the carbonate with barium hydroxide.

The malic dehydrogenase was prepared from *Micrococcus lysodeikticus*.<sup>1</sup> The bacteria were washed three times with cold acetone (4°), dried, and then washed six times with 50 volumes of 0.1 M phosphate buffer, pH 8.0. The centrifuged bacteria were then dried on a porous plate. This preparation oxidizes malate and fumarate but not succinate. Oxalacetate is oxidized slowly or not at all. The ability to oxidize oxalacetate is recovered, however, after extended incubation. Therefore, in converting malate to oxalacetate, the reaction was stopped when the carbon dioxide evolution approached 60 per cent of the oxygen uptake. The 30 ml. of reaction mixture contained 20 ml. of the centrifuged fumarate and malate mixture, obtained as described above, and 300 mg. of the bacterial preparation. The reaction time was

<sup>1</sup> Thanks are expressed to L. O. Krampitz for the preparation of this enzyme.

1.5 hours at 30°. The mixture was cooled immediately, acidified with citric acid, and aerated vigorously for 10 minutes to remove bound carbon dioxide. The citric acid-aniline was then added and the carbon dioxide formed from the oxalacetic acid was collected in alkali.

The acetaldehyde and succinic acid were converted to carbon dioxide by persulfate oxidation (5) for use in the mass spectrometer determination (4).

Data obtained by both methods of decarboxylating succinic acid are shown in Table I. The succinic acid was obtained from the fermentations of galactose and from pyruvate by *Escherichia*

TABLE I  
Distribution of Fixed Carbon in Succinic Acid

	C <sup>13</sup> in succinic acid	CH <sub>2</sub> carbon	COOH carbon		Calculated* C <sup>13</sup> in COOH
		C <sup>13</sup> in CH <sub>2</sub> CHO by KMnO <sub>4</sub> oxidation	C <sup>13</sup> in CO <sub>2</sub> by KMnO <sub>4</sub> oxidation	C <sup>13</sup> in CO <sub>2</sub> by citric acid-aniline method	
	per cent	per cent	per cent	per cent	per cent
<i>Escherichia coli</i> , galactose	1.46	1.13	1.88	1.84	1.83
<i>Escherichia coli</i> , pyruvate	1.24	1.11	1.38		1.39
<i>Propionibacterium pentosaceum</i> , glycerol	1.28	1.10	1.46	1.38	1.47

\* The following equation was used in the calculation:  $2X + 2 \times 1.09 = 4 \times \text{per cent C}^{13} \text{ in succinic acid}$ ;  $X = \text{per cent C}^{13} \text{ in the carboxyl group}$ .

*coli*, and from glycerol by *Propionibacterium pentosaceum*. In interpretation of the results it should be remembered that all natural carbon contains about 1.09 per cent C<sup>13</sup> and that the variation in the mass spectrograph analysis of C<sup>13</sup> is about  $\pm 0.02$ . The values in the first column of Table I show that the succinic acid contained fixed C<sup>13</sup> which was derived from the labeled carbon dioxide. The methylene carbon groups of the succinic acid obtained as the degradation product acetaldehyde, contained the natural complement of C<sup>13</sup>; therefore, there was no fixed carbon present in these positions. The carbon dioxide produced from the carboxyl carbon by both methods of decarboxylation contained C<sup>13</sup> in concentration well above the normal. It is, thus, evident that the fixed carbon is exclusively in the carboxyl groups of the

succinic acid. There is good agreement between the calculated values for the carboxyl carbon, which were estimated on the basis that all the fixed carbon is in the carboxyl group, and the experimental values.

It is not possible to prove by degradation of a symmetrical dicarboxylic acid that the fixed  $C^{13}$  is in only *one* of the carboxyl groups. This would be the case if the acid were formed according to our proposals from pyruvic acid and carbon dioxide. However, proof that the fixed carbon is limited to the carboxyl groups does offer considerable evidence of the validity of the proposed reaction, particularly because the quantitative results of Wood *et al.* (12) indicate that only one fixed carbon atom is in the succinic acid molecule. The fact that *Escherichia coli* forms succinic acid from pyruvic acid with fixation of carbon dioxide in the carboxyl groups lends weight to the suggested (9, 10) intermediary rôle of pyruvic acid in carbon dioxide assimilation.

#### SUMMARY

Proof has been obtained by degradation of succinic acid synthesized by bacteria that the fixed carbon dioxide, which has been labeled with  $C^{13}$ , is exclusively in the carboxyl groups of the acid. This observation is in agreement with the authors' proposal that carbon dioxide is fixed by union with pyruvic acid.

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## STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

### XII. AN ELECTROPHORETIC STUDY OF THE EFFECT OF ANTI-COAGULANTS ON HUMAN PLASMA PROTEINS, WITH REMARKS ON THE SEPARATION OF THE HEPARIN COMPLEMENT\*

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The manner in which the easily demonstrable anticoagulant effect of heparin on blood is brought about has been a matter of much conjecture. It is known that the heparin action is mediated through a factor, the heparin complement, which is present in the serum albumin fraction but is not identical with the crystalline albumin (1-3). Heparin requires this complement in order to prevent the clotting of fibrinogen by thrombin. The same heparin-protein system or a very similar one is responsible for the inhibition by heparin of the conversion of prothrombin to thrombin under the influence of the thromboplastic factor (4). Heparin has also been shown to have a disruptive action on the thromboplastic lipoprotein from lungs; it displaces the phosphatides contained in it, forming a heparin-protein complex which exhibits anticoagulant activity (5).

The high content of sulfuric acid groups confers on heparin a strong electronegative charge. This property together with its high molecular weight probably is responsible for certain reactions of heparin described in the literature; *viz.*, the displacement of the flocculation point of proteins (6, 7), the inhibition of the action of fumarase (8), and the "neutralization" of the hemolytic complement (9).

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The remarkable properties of heparin made it appear of interest to study its influence on the electrophoretic behavior of plasma proteins. For reasons which will be discussed later in this paper, the following compounds were likewise examined: chondroitin-sulfuric acid, cellulose disulfuric acid, germanin (Bayer 205), benzenesulfonic acid. The method of Tiselius (10) offers a valuable tool for the investigation under physiological conditions of the manner in which anticoagulants are transported in the blood stream. It certainly will also prove of importance for the study of other physiologically active substances.

#### EXPERIMENTAL

##### *Material*

*Heparin*—The sodium salt of highly purified heparin was used in all experiments. We are indebted to Hoffmann-La Roche, Inc., Nutley, New Jersey, for a number of experimental preparations. The samples were, previous to the conversion into the sodium salt, precipitated as benzidine salt (Preparations 1 to 3), or crystallized as barium salt (Preparations 4 to 6) (compare (11)). Analytical data will be found in Table I. Preparation 6 was a heparin sample obtained from the Connaught Laboratories, Toronto, which had 110 units per mg., according to the definition given by Best (12). All samples were tested for anticoagulant activity (13) and found to have at least the same potency as Preparation 6. In a number of cases somewhat higher activities were observed; *e.g.*, in Preparation 5, which had about 130 units per mg.

*Cellulose Disulfuric Acid*—This substance was used as the sodium salt, the preparation of which has been described previously (14).

*Chondroitinsulfuric Acid*—The barium salt of this substance, isolated from cartilage, was kindly given us by Dr. K. Meyer of this College. The sodium salt used in these experiments was prepared by dialysis of a 1 per cent solution of the barium salt in water, contained in a cellophane bag, against a 10 per cent solution of sodium sulfate for 20 hours. In this manner the barium sulfate could be made to flocculate completely. After centrifugation the solution was dialyzed against distilled water.

The other substances used were commercial preparations.

*Methods*

The electrophoresis experiments were carried out at 1.5° in the apparatus described by Tiselius (10). For analysis the optical arrangement of Longworth (15) was used in most experiments. In several cases the optical method of Philpot and Svensson (16) was employed. The two methods gave identical results. In some of the separation experiments, the experimental arrangement of which will be discussed later in this paper, the separated

TABLE I  
*Heparin Preparations*

Preparation No.	Analysis		Electrophoresis				
	N	S	Buffer	pH	Mobility*  $u \times 10^6$	Area†	
						Descending side	Ascending side
	<i>per cent</i>	<i>per cent</i>					
1	2.2	11.3	0.025 M barbiturate	7.90	23.5		
2	2.0	11.6					
3	2.2	12.7	0.02 " phosphate	7.37	21.3	1	1
4	2.0	11.9	0.02 " "	7.37	21.6	1.3	* 1
5	2.3	12.0	0.02 " "	7.49	19.5	1.1	1
6	2.7	12.4					

\* The mobilities were calculated on the basis of the descending boundaries with the exception of Preparation 1, the mobility of which was computed from the ascending pattern.

† The area is reported in arbitrary units, as measured by planimeter readings. The area covered by the heparin peak on the ascending side is taken as 1 in all experiments.

components were again subjected to electrophoretic analysis. This was done in a micro apparatus which consisted of a tall single sectional cell holding 2 cc. of solution.

The following buffer solutions were used in the experiments under discussion: (1) 0.02 M phosphate buffer, (2) 0.02 M phosphate buffer containing 0.15 M NaCl, (3) 0.025 M barbiturate buffer. All protein preparations were diluted with and dialyzed against the buffer for 2 to 6 days, until equilibrium was reached. In several cases the presence of heparin in the protein solutions rendered the establishment of a satisfactory equilibrium quite

difficult. The protein concentrations employed in the various experiments will be found later in the paper. The oxalate plasma samples used were diluted with 2 or 3 parts of buffer prior to dialysis.

The anticoagulants examined were in most cases weighed into the protein solution before dialysis. Unless otherwise noted, they were present in 0.4 per cent concentration.

### *Experiments with Heparin*

*Heparin*—When heparin alone was subjected to electrophoresis, it moved as a single component with sharp boundaries towards the anode. The shape and size of the peaks on the descending and ascending sides were fairly symmetrical. There was no indication of the presence of a contaminating substance migrating towards the cathode, as described by Wilander (17). The data obtained with a number of heparin preparations are included in Table I.

*Plasma*—The electrophoretic pattern of a sample of normal human plasma is reproduced in Fig. 1.<sup>1</sup> The patterns resulting from the addition to normal plasma of heparin Preparations 1 and 5 are shown in Figs. 2 and 3 respectively. In a number of experiments of this type in which heparin Preparations 2 and 3 were likewise used it was found that the presence of heparin in a plasma had the following effects on its electrophoretic pattern. On the descending side the albumin peak was considerably broadened with the appearance, in most cases, of a new component (designated as the C component) which migrated somewhat faster than the albumin. In the globulin region the main effect was the disappearance of the “ $\beta$ -globulin disturbance;” *i.e.*, the tall sharp spike which normally accompanies the  $\beta$ -globulin boundary (19). On the ascending side the albumin peak was similarly broadened without the appearance of a separate new component. The globulins were broken up into a large number of small components. The mobilities of the various components in several typical experiments are given in Table II, Experiments 1, 2, 4, 5.

<sup>1</sup> The various fractions shown in the figures are marked with the following symbols: heparin = *He*; C component = *C*; albumin = *A*; globulins = *G*l or  $\alpha$ ,  $\beta$ ,  $\gamma$ ; fibrinogen =  $\phi$ . A discussion of the nature of the anomalous  $\delta$  and  $\epsilon$  boundaries will be found in a paper by Longworth and MacInnes (18). All patterns reproduced in this paper are actual photographs.

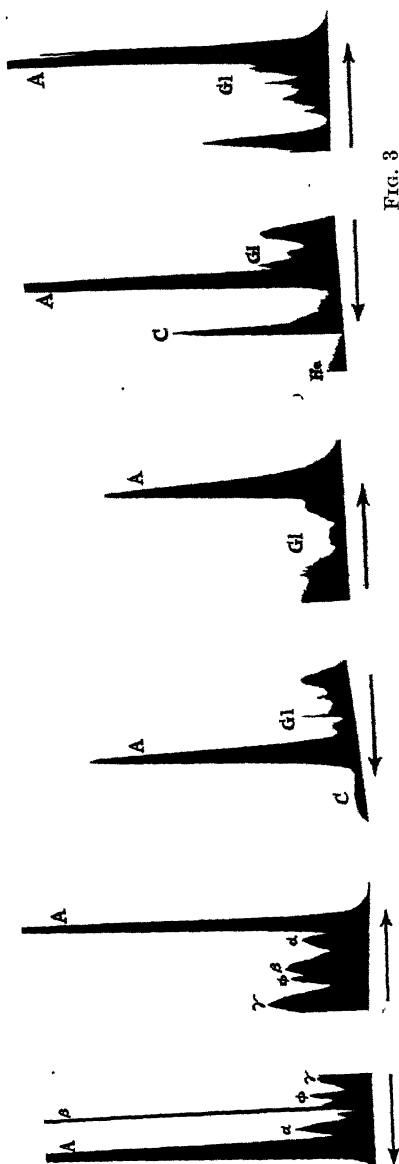


FIG. 1

FIG. 1. Plasma, 0.025 M barbiturate buffer (pH 7.76), after 1 hour. Left, descending pattern; right, ascending pattern. See footnote 1 for an explanation of the symbols.

FIG. 2. Plasma containing 0.4 per cent heparin (Preparation 1), 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.37), after 3 hours. Left, descending pattern; right, ascending pattern.

FIG. 3. Plasma containing 0.4 per cent heparin (Preparation 5), 0.025 M barbiturate buffer (pH 7.58), after 45 minutes. Left, descending pattern; right, ascending pattern.

FIG. 2

FIG. 2. Plasma containing 0.4 per cent heparin (Preparation 1), 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.37), after 3 hours. Left, descending pattern; right, ascending pattern.

FIG. 3. Plasma containing 0.4 per cent heparin (Preparation 5), 0.025 M barbiturate buffer (pH 7.58), after 45 minutes. Left, descending pattern; right, ascending pattern.

FIG. 3. Plasma containing 0.4 per cent heparin (Preparation 5), 0.025 M barbiturate buffer (pH 7.58), after 45 minutes. Left, descending pattern; right, ascending pattern.

The effect of heparin generally was more pronounced in buffers which did not contain sodium chloride. In the course of the experiments two heparin samples (Preparations 4 and 6 in Table I) were encountered which behaved somewhat differently. They did not produce a new component on the descending side. Instead, the heparin peak preceding the albumin showed very marked lack of symmetry between the descending and ascending patterns: it was many times larger on the descending side. These findings will be more fully discussed later in this paper. An experiment of this type is given as Experiment 3 in Table II.

TABLE II  
*Effect of Heparin on Electrophoretic Pattern of Human Plasma*

Experiment No.	Heparin preparation No.*	Buffer	pH	Mobilities† ( $\mu \times 10^5$ )		
				Heparin	C component	Albumin
1	1	0.02 M phosphate + 0.15 M NaCl	7.40	13.9	7.7	4.8
2	1	“ “	7.37	13.3	9.3	4.8
3	4	“ “	7.40	14.1		4.9
4	5	0.025 M barbiturate	7.58	17.7	11.4	5.0
5	5	0.025 “ “	7.79	21.3	13.3	6.1

\* The numbers refer to Table I. The heparin concentration used was 0.4 per cent.

† The mobilities were calculated on the basis of the descending boundaries.

*Dialyzed Plasma*—In a number of experiments human plasma was used which had been freed of salts by dialysis against running water for 2 days followed by dialysis against distilled water for 5 hours. In this manner, a large portion of the globulins precipitated and was removed by centrifugation. The electrophoresis pattern of dialyzed plasma is reproduced in Fig. 4. An experiment with heparin Preparation 3 in which the production of the new C component on the descending side was particularly clear is reproduced in Fig. 5. Heparin Preparations 4 and 6 again failed to produce a separate new component, but the patterns showed the same marked dissymmetry which was mentioned in the preceding paragraph. A typical experiment of this type is

shown in Fig. 6. The electrophoretic data for a number of experiments with dialyzed plasma are summarized in Table III. The degree of dissymmetry between the descending and ascending patterns can be gathered from the comparative measurements of the areas covered by the various peaks, likewise indicated in Table III.

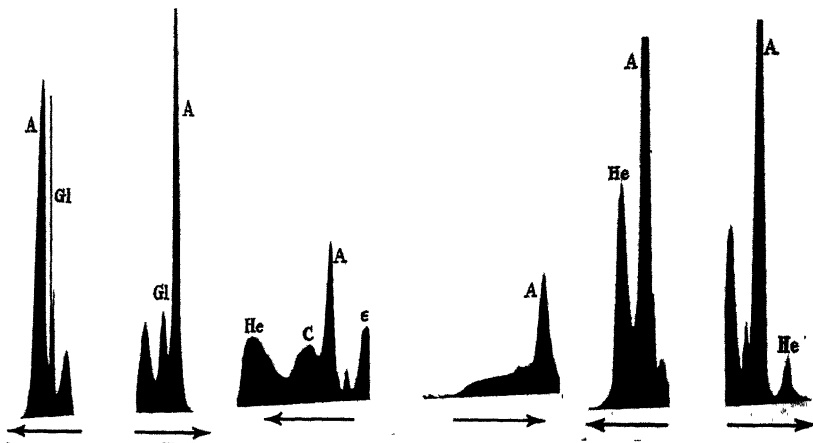


FIG. 4

FIG. 5

FIG. 6

FIG. 4. Dialyzed plasma, 0.02 M phosphate buffer (pH 7.43), after 20 minutes. Left, descending pattern; right, ascending pattern. See foot-note 1 for an explanation of the symbols.

FIG. 5. Dialyzed plasma containing 0.4 per cent heparin (Preparation 3), 0.02 M phosphate buffer (pH 7.40), after 45 minutes. Left, descending pattern; right, ascending pattern.

FIG. 6. Dialyzed plasma containing 0.4 per cent heparin (Preparation 4), 0.02 M phosphate buffer (pH 7.43), after 17 minutes. Left, descending pattern; right, ascending pattern.

*Albumin Preparations from Plasma*—Heparin is known to require the presence of plasma albumin or of one component of the albumin fraction for its anticoagulant action (1-3). It was, for this reason, of interest to study the effect of heparin on the electrophoretic pattern of various albumin preparations.

The patterns obtained with an albumin preparation from human plasma prepared by two precipitations at 75 per cent saturation with ammonium sulfate are shown in Fig. 7 (in absence of heparin)

TABLE III

*Effect of Heparin on Electrophoretic Pattern of Dialyzed Human Plasma*

Experiment No.	Heparin preparation No.*	pH†	Mobilities‡ ( $u \times 10^5$ )			Area§				
			Heparin	C component	Albumin	Descending side			Ascending side	
						Heparin	C component	Albumin	Heparin	Albumin
1	1	7.43	14.0	8.3	5.8	4.2	3.4	3.7	1	6.6
2	3	7.40	14.2	7.4	4.7	3.1	1.9	2.2	1	4.3
3	3	7.42	18.2	9.9	6.0	3.2	2.6	1.7	1	5.9
4	4	7.43	14.8		5.8	22.6		33.6	1	35.8
5	6	7.28	19.7		7.3	4.3		4.3	1	4.2

\* The numbers refer to Table I. The heparin concentration used was 0.4 per cent.

† In all experiments 0.02 M phosphate buffer was used.

‡ The mobilities were calculated on the basis of the descending boundaries.

§ The area is reported in arbitrary units, as measured by planimeter readings. The area covered by the heparin peak on the ascending side is taken as 1 in all experiments.

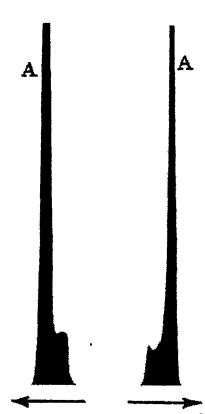


FIG. 7

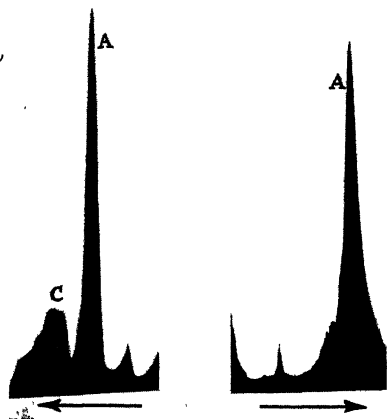


FIG. 8

FIG. 7. Albumin, precipitated at 75 per cent ammonium sulfate saturation, 0.025 M barbiturate buffer (pH 7.78), after 30 minutes. Left, descending pattern; right, ascending pattern. See foot-note 1 for an explanation of the symbols.

FIG. 8. Albumin, precipitated at 75 per cent ammonium sulfate saturation, containing 0.4 per cent heparin (Preparation 1), 0.025 M barbiturate buffer (pH 7.76), after 90 minutes. Left, descending pattern; right, ascending pattern.

and in Fig. 8 (in presence of heparin). It can be seen that albumin prepared in this manner contains a small amount of globulin. The details of this experiment are given as Experiment 1 in Table IV. Fig. 8 clearly shows the new C component created by the

TABLE IV  
*Effect of Heparin on Electrophoretic Pattern of Albumin Preparations from Human Plasma*

Experiment No.	Albumin*	Concentration of albumin Heparin preparation No.†		Buffer	pH	Mobilities‡ (u×10 <sup>5</sup> )				Relative area of C components
						Without heparin		With heparin		
						Albumin	Heparin	C component	Albumin	
		per cent								per cent
1	75%	2.3	1	0.025 M barbiturate	7.77	6.1	20	9.2	5.5	36
2	Albumin (Howe)	1.1	1	0.02 M phosphate + 0.15 M NaCl	7.41	4.9	15	7.1	4.4	27
3	“ “	1.7	1	0.06 M phosphate	7.78		18	10.9	6.3	
4	“ “	2.1	4	0.02 “ “	7.46		18		6.9	
5	Crystalline albumin	1.2	1	0.02 “ “ + 0.15 M NaCl	7.34	5.3	16	10.4	5.2	15
6	“ “	1.0	1	“ “	7.4		14	8.9	4.3	

\* 75 per cent represents the plasma fraction insoluble at this ammonium sulfate concentration. The crystalline albumin from human plasma was a preparation of Dr. F. E. Kendall.

† The numbers refer to Table I.

‡ The mobilities were calculated on the basis of the descending boundaries.

§ The relative area of the C component is given as per cent of the total area covered by the albumin and C components, as measured by planimeter readings.

addition of heparin moving faster than the albumin boundary. In experiments in which isolated albumin preparations were used it was generally observed that the C component was more clearly discernible on the descending than on the ascending side which usually showed only a broadening of the albumin peak.



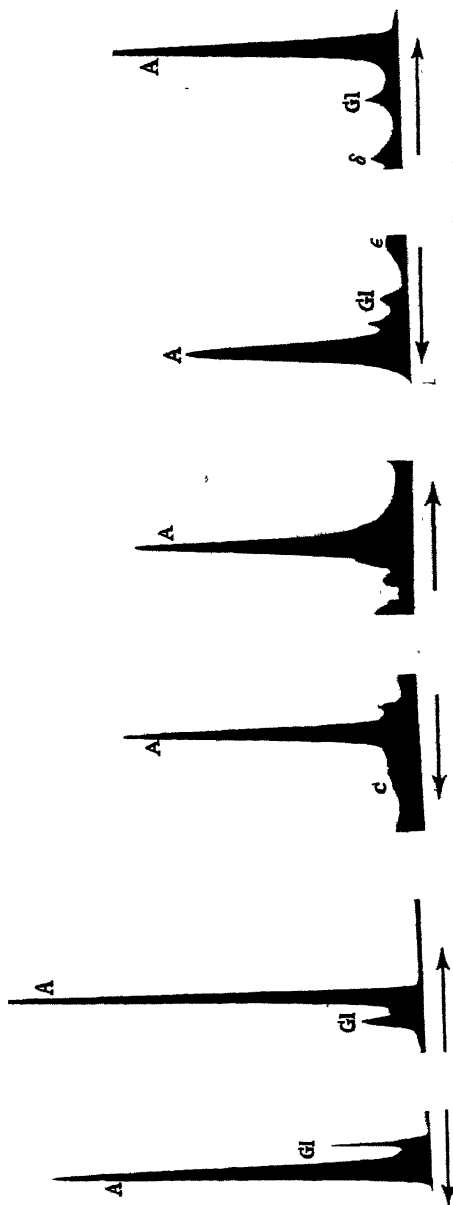


FIG. 11

FIG. 10

FIG. 9

FIG. 9. Albumin (according to Howe (20)), 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.41), after 2 hours. Left, descending pattern; right, ascending pattern. See foot-note 1 for an explanation of the symbols.

FIG. 10. Albumin (according to Howe (20)) containing 0.4 per cent heparin (Preparation 1), 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.41), after 2 hours. Left, descending pattern; right, ascending pattern.

FIG. 11. Albumin (Howe) and heparin (same as in Fig. 10), after removal of heparin by means of salmine, 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.28), after 3 hours. Left, descending pattern; right, ascending pattern.

Similar results were obtained with albumin prepared from human plasma by the method of Howe (20). The types of pattern observed are shown in Fig. 9 (in absence of heparin) and in Fig. 10 (in presence of heparin). The presence of a small amount of globulin (mobility 2.9 at pH 7.8) in the albumin preparation is evident from Fig. 9. Details of such experiments are given in Table IV, Experiments 2 and 3. Heparin Preparation 4 again failed to produce the C component, but the heparin peak appearing on the descending side was many times larger than that on the ascending side, as was mentioned before (Experiment 4 in Table IV).

It has been demonstrated in this laboratory (21) that the anti-coagulant action of heparin both *in vivo* and *in vitro* is destroyed by the basic protamine salmine. It was of interest to ascertain whether the effect of heparin on plasma albumin was equally reversible. Heparin (Preparation 1) in a concentration of 0.4 per cent was added to a 1.1 per cent solution of an albumin preparation obtained according to Howe (20), as mentioned in the preceding paragraph. The addition of 50 mg. of salmine sulfate to 15 cc. of this heparin-albumin mixture produced immediate precipitation of the salt between heparin and salmine which was removed by centrifugation after dialysis of the mixture against several changes of 0.02 M phosphate buffer of pH 7.28 (containing 0.15 M sodium chloride) for 2 days. The electrophoretic pattern (Fig. 11) showed no evidence of a new component faster than the albumin boundary (mobility 5.0).

Samples of crystalline albumin from human plasma, prepared by Dr. F. E. Kendall of the Research Division for Chronic Diseases, Welfare Island, New York, were likewise examined (compare (22)). This substance, as was recently shown (3), does not complement the inhibiting effect of heparin on the clotting of fibrinogen by thrombin. The crystalline albumin was electrophoretically completely homogeneous. Heparin created a small component on the descending side migrating more rapidly than the main albumin spike. The pattern obtained is reproduced in Fig. 12. Other details will be found in Table IV, Experiments 5 and 6.

*Globulin*—A control run with an isolated globulin preparation

appeared of interest. A highly purified globulin fraction from human serum (designated Antigen 1) was kindly placed at our disposal by Dr. F. E. Kendall. This protein migrated as a single component with the mobility of a  $\gamma$ -globulin. A 0.6 per cent solution in 0.02 M phosphate buffer of pH 7.38 gave a mobility of 1.0. The same preparation in the presence of 0.4 per cent heparin (Preparation 1) at pH 7.41 showed the following mobilities: heparin 14.0, globulin 0.5. Heparin had no effect on the globulin boundary.

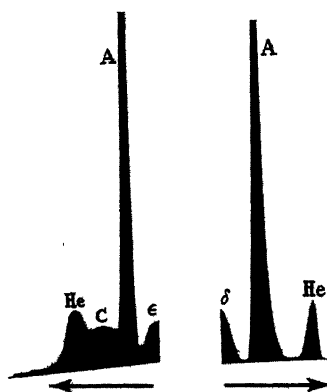


FIG. 12. Crystalline albumin (Kendall) containing 0.4 per cent heparin (Preparation 1), 0.02 M phosphate buffer + 0.15 M NaCl (pH 7.4), after 1 hour. Left, descending pattern; right, ascending pattern. See foot-note 1 for an explanation of the symbols.

*Viscosity Effect*—In order to exclude the possibility of a viscosity effect in the formation of the C component by heparin, a few viscosity measurements were carried out by means of an Ostwald viscosimeter. Heparin, in the concentration used in the electrophoresis experiments, had practically no effect on the viscosity of the protein solutions. Dialyzed human plasma diluted with 2 parts of 0.02 M phosphate buffer of pH 7.82 had a relative viscosity of 1.13 at 5°. The relative viscosities observed under identical conditions in presence of 0.4 per cent heparin (Preparations 3 and 4) were 1.24. Similar results were obtained with the other protein and buffer solutions employed in this work.

*Separation Experiments*

In a number of experiments with heparin and several albumin preparations obtained according to Howe (20) an electrophoretic separation into three fractions was performed. All experiments were carried out with the compensation arrangement in which a

TABLE V  
*Heparin Complement Activity of Albumin Fractions Separated by Electrophoresis*

Experiment No.	Heparin preparation No.*	Buffer	pH	Components	Heparin complement activity										
					N per cc. albumin solution tested	Clotting time, sec.†									
						30	40	65	90	120	180	600	1200	1500	1800
					mg.										
1	1	0.025 M barbiturate	7.78	Fast	1.0	—	—	—	—	—	—	—	—	—	—
				Slow	1.0	+	—	—	—	—	—	—	—	—	
				Control		+									
2	1	0.06 M phosphate	7.35	Fast	0.25				—	—	—	—	—	—	
				Middle	0.25				—	—	—	—	—	—	
				Slow	0.37					+	—	—	—	—	
				Control					+						
3	2	0.06 “ “	7.37	Fast	0.52		—	—	—	—	—	—	—	—	
				Middle	0.52		—	—	—	—	—	—	—	—	
				Slow	0.52		—	—	+	—	—	—	—	—	
				Control			+								
4	4	0.02 “ “	7.42	Fast	0.24		—	—	—	—	—	—	—	—	
				Middle	0.24		—	—	—	—	±	±	±	±	
				Slow	0.44		—	—	±	+					
				Control			+								

\* The numbers refer to Table I.

† + = clot; ± = incomplete clot; — = no clot.

counter flow opposite to the direction of the migrating proteins was produced by means of a clockwork and plunger. In one of the experiments (No. 3 in Table V) a macro cell holding 100 cc. was used which consisted of two tall sections each 96 mm. high with a cross-section of 50 × 8 mm. Each channel had twice the length and half the width of the original separation cell as designed by

Tiselius (23). This reduction in channel width allows a much higher voltage to be impressed across the cell without disturbance due to heat convection. The longer sections also have the advantage of allowing greater visibility, since the boundaries are not obscured by the flange plates. The separated components may be removed by means of a long capillary tube.

The separated fractions (fast, middle, slow), the approximate distribution of which is indicated in Fig. 13, *a*, were examined for heparin complement activity by means of the clotting system

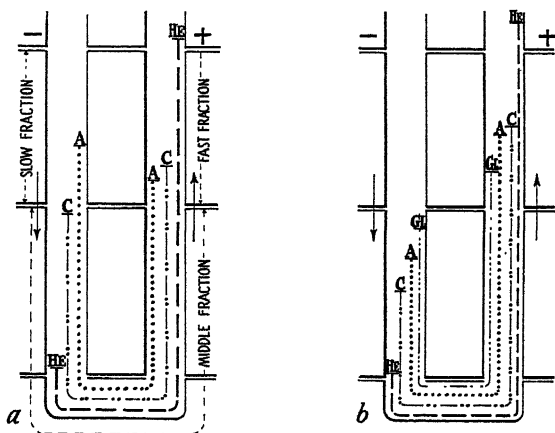


FIG. 13. Relative position of boundaries (*a*) in separation experiments with albumin fractions; (*b*) in electrophoresis of plasma containing heparin. See foot-note 1 for an explanation of the symbols.

(human fibrinogen, human thrombin, heparin) previously described (3). As shown in the diagram, the fast and middle fractions contained the C component and albumin in addition to heparin; the slow fraction comprised albumin together with what little globulin had been present as impurity in the original protein preparation.

The experimental results are summarized in Table V. In all cases a 5.2 per cent albumin solution was mixed with 2 parts of buffer (free of sodium chloride) and dialyzed against the buffer until the Donnan equilibrium was established. Heparin was then added in a concentration of 0.4 per cent. The electrophoresis was carried out until proper separation had taken place. In

Experiments 1, 2, and 4 (Table V) 15 cc. of protein solution were subjected to an electric field of 8 volts per cm. in the standard cell for about 5 hours. Experiment 3 was carried out with 100 cc. of solution in the new macro cell described above for about 20 hours at a field strength of 6.2 volts per cm.

In Experiment 1 in which barbiturate buffer had been used, the separated fractions were dialyzed against tap water for 2 days and against saline for 1 day before testing; the other solutions were tested as obtained. In each run all fractions were adjusted to the same nitrogen concentration. As was pointed out in a previous paper (3), there exists a critical relationship between the anticoagulant activity of heparin and the relative concentration of heparin complement and thrombin. For this reason the thrombin concentration had to be so chosen as to allow differentiation between the fractions tested. Very strong thrombin solutions produced clots in all cases, as was to be expected.

It will be seen in Table V that the fast and middle components invariably exhibited heparin complement activity, whereas the slow components were inactive. The clotting system consisted of a mixture of 0.1 cc. of human thrombin solution, 0.06 cc. of a 0.3 per cent heparin solution, and 0.06 cc. of the protein fraction tested, to which 3 minutes later 0.2 cc. of human fibrinogen was added. The substances were all dissolved in physiological saline. The tests were carried out at room temperature. The following control experiments not incorporated in Table V were likewise carried out. As shown in Fig. 13, *a*, the fast and middle components, as obtained from the electrophoresis cell, necessarily contained a small amount of heparin in addition to the standard amount of heparin added in the clotting test. However, doubling the concentration of heparin did not change the inactivity of the slow components. On the other hand, the slow fractions always contained small amounts of globulin (7 to 9 per cent according to electrophoretic analysis); but the results remained unchanged when the inactive slow fractions were tested in concentrations 50 per cent higher than those of the active fast and middle components.

In several cases electrophoretic patterns of the separated fractions were obtained. The slow fraction of Experiment 1 after being tested was dialyzed against barbiturate buffer of pH 7.78. Heparin was added in a concentration of 0.4 per cent, and 2 cc.

of this protein solution (containing 0.7 per cent albumin) were subjected to electrophoresis in the micro cell. The electrophoretic pattern showed three components with the following mobilities: heparin 18.8, C component 8.4, albumin 5.0.

Both the fast and slow components obtained in Experiment 3 were again subjected to electrophoretic analysis without added heparin. The electrophoresis was carried out in 0.06 M phosphate buffer (free of sodium chloride) at pH 7.35. The fast fraction (containing 0.36 per cent protein) showed a trace of the C component and a sharp albumin spike (mobility 5.5). The slow fraction (containing 0.50 per cent protein) only exhibited a sharp albumin peak (mobility 5.6).

*Experiments with Other Sulfuric Acid Esters and Sulfonic Acids*

*Germanin (Bayer 205)*—The anticoagulant effect of germanin has been known for some time (24). Electrophoresis experiments with plasma containing 0.3 per cent germanin produced patterns essentially similar to those obtained with heparin (Table VI, Experiment 1). A new component moved ahead of the albumin peak. Germanin caused the plasma to become somewhat opaque during the electrophoresis because of the formation of a slight precipitate. For this reason some of the boundaries were obscured.

*Cellulose Sulfuric Acid*—The sodium salt of this compound which exhibits markedly anticoagulant properties (14) effected the formation of the fast C component similar to heparin and germanin (Table VI, Experiments 2 and 3). In these experiments, as in the ones with germanin, the appearance of a precipitate made the observation of the boundaries difficult. This was not improved by the substitution of serum for plasma.

*Chondroitinsulfuric Acid*—In experiments in which human plasma or dialyzed plasma, containing 0.06 per cent of sodium chondroitinsulfate, was used, completely normal electrophoresis patterns were obtained. There was no indication of the formation of the fast C component. The mobilities of the components are given in Table VII, Experiments 1 and 2.

*Benzenesulfonic Acid*—The sodium salt of this compound, used as a control substance of low molecular weight, did not influence

TABLE VI  
*Effect of Synthetic Anticoagulants on Electrophoretic Pattern of Human Plasma*

Experiment No.	Anticoagulant	Concentration of anticoagulant	Buffer	pH	Mobilities ( $u \times 10^5$ )		
					Anticoagulant	Component	Albumin
1	Germanin (Bayer 205)	0.3	0.02 M phosphate + 0.15 M NaCl	7.35	13.3	11.2	5.2
2	Cellulose sulfuric acid	0.4	" "	7.38	14.9*	8.7*	5.0*
3	" "	0.4	0.06 M phosphate + 0.15 M NaCl	7.34	13.8	8.7*	4.9

The mobilities were calculated on the basis of the descending boundaries with the exception of the values indicated by the asterisk, which were derived from the ascending pattern. In Experiment 2 serum was substituted for plasma.

TABLE VII  
*Effect of Non-Anticoagulant Substances on Electrophoretic Pattern of Human Plasma*

Experiment No.	Substance	Plasma	Buffer	pH	Mobilities* ( $u \times 10^5$ )					
					Substance added	Albumin	Globulins			Fibrinogen
							$\alpha$ -	$\beta$ -	$\gamma$ -	
1	Chondroitinsulfuric acid	Normal	0.02 M phosphate + 0.15 M NaCl	7.38	13.6	5.2	3.8	3.0	0.8	2.0
2	" "	Dialyzed	0.02 M phosphate	7.37	17.3	6.0				
3	Benzenesulfonic acid	Normal	0.02 M phosphate + 0.15 M NaCl	7.36	14.1	4.6	3.4	2.4	0.8	1.8

\* The mobilities were calculated on the basis of the descending boundaries.

the normal electrophoresis pattern of human plasma to which it had been added in a concentration of 0.4 per cent (Table VII, Experiment 3).



## DISCUSSION

The existence of a heparin complement in the plasma albumin fraction (1-3) suggested the study of the electrophoretic behavior of plasma proteins in the presence of heparin. The results presented in this communication show that anticoagulants of the heparin type react with both the plasma albumin and globulin fractions. Most heparin samples examined caused the appearance in plasma and isolated plasma albumin preparations of a new boundary (the C component) with a mobility intermediate between that of heparin and of albumin. The globulin patterns appeared broken up into a large number of small components.

In the course of this work it was repeatedly noticed that two heparin samples (Preparations 4 and 6 in Table I) failed to produce the new component. It was, however, observed that experiments with these heparin preparations yielded highly asymmetric electrophoresis patterns: the heparin peaks on the descending side, *i.e.* on the side which in the presence of the other heparin samples showed the C component, were many times larger than on the ascending side. No cogent reason for this anomalous behavior can be given at present.

A consideration of the influence of heparin on the electrophoresis pattern of plasma albumin will have to start from the fact that heparin, in order to prevent the clotting of fibrinogen by thrombin, requires the presence of albumin. It is important to decide whether this heparin complement is identical with, or contained in, the C component here described. Apart from indirect evidence for this identity, *e.g.* the inability of substances devoid of anticoagulant activity to produce the new component, more conclusive proof is afforded by the separation experiments reported in this paper. In these experiments the contents of the cell after prolonged electrophoresis were divided into three parts, the fast, middle, and slow components (compare Fig. 13, *a*). When tested in the presence of added heparin for heparin complement activity under strictly comparable conditions, the fast and middle fractions were very markedly active, while the slow fraction was inactive.

This finding perhaps has some bearing on the question of the homogeneity of plasma albumin. It apparently indicates that the electrophoretically homogeneous albumin is not so in other

respects.<sup>2</sup> The heparin complement, although not different from albumin in electrophoretic mobility in the absence of heparin, obviously is a specific component of the albumin fraction. This, of course, does not mean that the appearance under the influence of heparin of a component intermediate in mobility between albumin and heparin in itself implies the formation of the physiological anticoagulant complex. The natural clotting inhibitor, presumably is an albumin compound in which heparin serves as a prosthetic group. But it seems that even albumin preparations which do not support the anticoagulant action of heparin may still be able to combine with it. The experiments with crystalline albumin from human serum which does not complement heparin (3) may serve as an example. It will be of interest to determine whether the heparin complement is concentrated in the mother liquors from the crystallization of the albumin or whether chemical changes in the crystalline albumin account for its inactivity as heparin complement.

The reaction of heparin with the globulins observed in the plasma experiments probably is best brought into parallel with the disruptive action of heparin on the thromboplastic lipoprotein from lungs previously reported from this laboratory (5). This reaction has been shown to involve the liberation of bound lipids. In the present experiments the most likely point of attack is the extremely labile  $\beta$ -globulin fraction which is known to carry the plasma lipids with it (19, 23, 26, 27). A globulin fraction moving with the mobility of  $\gamma$ -globulin was not attacked by heparin.

The C component, as was pointed out before, in all probability is a compound between heparin and an albumin component. This complex is dissociable, as shown (a) by the larger relative area covered by the peak of the C component in buffers free of sodium chloride than in those containing it;<sup>3</sup> (b) by the lack of symmetry between the electrophoresis patterns obtained on the descending and ascending sides; (c) by the disappearance of the C component

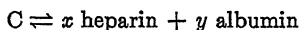
<sup>2</sup> At a low pH (pH 4.0) serum albumin was found to exhibit two boundaries in the electrophoresis apparatus (25).

<sup>3</sup> For the effect of salt concentration on the formation of complexes between proteins and nucleic acid and of antigen-antibody precipitates compare the data of Hammarsten and Hammarsten (28) and Heidelberger *et al.* (29).

from plasma which had been freed of previously added heparin by means of salmine. The latter finding shows in a particularly convincing manner that the reaction between heparin and albumin is reversible.

The dissociation of the C component must proceed at a finite rate, since otherwise no separate moving boundary could have been observed. The type of binding (polar or non-polar) that might occur between heparin and albumin is at present entirely a matter of conjecture. The experiments were carried out at a pH well above the isoelectric point of albumin.

The lack of symmetry between the patterns observed for the descending and ascending boundaries is very marked.<sup>4</sup> Fig. 13, *b* gives a schematic illustration of the position of the various boundaries in plasma containing heparin. The globulin boundaries are in contact with heparin on the ascending side only, and that is where the reaction between plasma globulins and heparin was noticed. The boundaries of the C component were much more clearly discernible on the descending than on the ascending side. An exact treatment of the reactions occurring in an electric field in a system of the type



appears hazardous and will not be attempted here. An inspection of Fig. 13, *b* will make it clear that the boundary of the C component has no contact with heparin on the descending side and no contact with albumin on the ascending side. It is understandable that the amounts of heparin and protein available for combination, provided they combine stoichiometrically to form the C component, will be of influence on its degree of stability on the descending and ascending sides during electrophoresis.

The synthetic anticoagulants known so far have at most about 6 per cent of the anticoagulant potency of pure heparin. With the exception of the agents which act by removing calcium from the clotting system they are all polysulfuric or polysulfonic acids. It is probable that some of the acidic groups of the anticoagulant

<sup>4</sup> The experiments presented in this paper involve the interaction between an acidic polysaccharide and a protein. A discussion of abnormal pattern asymmetries as indication of protein-protein interaction will be found in the paper by Longsworth *et al.* (30).

molecule serve to arrange albumin molecules around it.<sup>5</sup> It cannot yet be decided whether a certain steric arrangement of the sulfuric acid groups in the anticoagulant is decisive for the degree of its potency.

The results obtained with germanin (Bayer 205) were of interest, since they showed that this drug, which is known to be retained in the body for a long time, migrates, at least in part, with the albumin fraction. In this connection reference should be made to the work of Bournsnel and Wormall (32) which by means of salting-out experiments demonstrated the combination of germanin with plasma proteins.

The authors wish to express their appreciation to Dr. L. G. Longsworth of The Rockefeller Institute for Medical Research for helpful discussion of the experimental results, to Dr. F. E. Kendall of the Research Division for Chronic Diseases, Welfare Island, New York, for specimens of crystalline albumin from human serum, and to Miss Joan Korach and Miss Sheila Goldsmith for technical assistance. They are indebted to Hoffmann-La Roche, Inc., Nutley, New Jersey, for a number of heparin preparations.

#### SUMMARY

The electrophoresis patterns resulting from the addition of heparin and other anticoagulants to human plasma, dialyzed plasma, and various albumin preparations from human plasma were studied. Most heparin samples examined and synthetic anticoagulants (cellulose sulfuric acid, germanin) caused the appearance of a new electrophoretic component (the C component) which migrated with a mobility intermediate between that of heparin and albumin. There was also evidence for a reaction between heparin and the plasma globulins. Substances devoid of anticoagulant activity, like chondroitinsulfuric acid and benzene-sulfonic acid, failed to change the normal electrophoresis pattern of plasma.

The natural clotting inhibitor is considered as a complex be-

<sup>5</sup> In ultracentrifuge experiments von Mutzenbecher (31) found no increase of the molecular weight of serum albumin in the presence of heparin. It would be of interest to repeat these experiments with a highly purified heparin preparation.

tween heparin and a component of the plasma albumin fraction, the heparin complement. By means of electrophoretic separation experiments in which plasma albumin preparations were, in the presence of heparin, divided into three fractions, it could be shown that the fast and middle albumin fractions in contrast to the slow fraction contained the heparin complement. Some of the theoretical implications of these findings are discussed.

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## THE METABOLISM OF SULFUR

### XXIX. S-CARBOXYMETHYLCYSTEINE

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The working hypothesis of Brand and his associates (1) for the conversion of methionine to cysteine was based upon experiments in which S-carboxymethylcysteine was administered orally to both normal and cystinuric humans. They suggested that thioglycolic acid was first formed from S-carboxymethylcysteine and that this thiol derivative reacted with cysteine to form a mixed disulfide which was excreted in the urine. The existence of such a complex was postulated on the basis of the partition of urinary sulfur and on various color reactions of the urine, before and after reduction with zinc and hydrochloric acid. Isolation of the complex was not accomplished. They believed that part of the S-carboxymethylcysteine was excreted unchanged, part was oxidized and its sulfur excreted as inorganic sulfate, and part excreted as an intermediate which contained sulfur in a disulfide linkage; *i.e.*, the mixed disulfide of cysteine and thioglycolic acid.

In view of the importance of these observations, we have investigated the problem further with a different species, the rabbit, which has been used commonly in studies of sulfur metabolism. It has been possible also to observe the results obtained after parenteral administration of the compound. As has been pointed out (2-4), possible changes by the action of the intestinal microflora must be considered when the sulfur compounds are administered orally. This makes it highly desirable to study the results of parenteral administration in which the possibility of such secondary changes is ruled out.

S-Carboxymethylcysteine has been prepared (5, 6) by the reaction of cysteine hydrochloride with the monohalogen derivatives



of acetic acid. A more direct synthesis, which does not require the use of cysteine hydrochloride, difficult to obtain in a pure state, was accomplished by the action of sodium in liquid ammonia on cystine, followed by condensation with monochloroacetic acid. After the reduction of the cystine, carried out as described by du Vigneaud, Audenrieth, and Loring (7), slightly more than the theoretical amount of redistilled monochloroacetic acid was added slowly and after the reaction was completed the ammonia was allowed to evaporate at room temperature. The further treatment of the residue was as described by Michaelis and Schubert (6). A yield of approximately 55 per cent was obtained.

The recrystallized compound contained 7.78 per cent nitrogen and 17.75 per cent sulfur (theoretical, 7.83 and 17.88 per cent respectively), and melted at 191–192° (corrected). A sample of S-carboxymethylcysteine prepared by Dr. Erwin Brand<sup>1</sup> also melted at 191–192°. This is somewhat higher than the melting points previously recorded by Michaelis and Schubert (6) and Dickens<sup>2</sup> (5).

Male rabbits of 2 to 4 kilos in weight, maintained on constant weighed diets of a commercial rabbit chow and cabbage, were used as subjects, the general conduct of the experiments being uniform with that of previous similar experiments in this laboratory as were the methods of analysis (8). Ether-soluble sulfur was determined as the total sulfur extractable from the acidified urine by ether in an extraction apparatus of the type used by Griffith (9). The urine was made acid to litmus with hydrochloric acid. 1 cc. of a 2 per cent solution of sodium tungstate was added to prevent emulsification during the extraction. Disulfide linkages were determined by the method of Shinohara and Padis (10) and of Folin and Marenzi as modified by Rimington (11).

The distribution of the extra sulfur in the urine following administration of S-carboxymethylcysteine is summarized in Table I. A slight oxidation of the sulfur of the compound to sulfate after

<sup>1</sup> We wish to express our indebtedness to Dr. Erwin Brand of Columbia University who generously placed at our disposal the S-carboxymethylcysteine used in our preliminary experiments.

<sup>2</sup> Dickens (5) first reported a low melting point of 84° which was later corrected to 184°. Michaelis and Schubert were apparently not aware of this correction.

feeding is suggested in certain experiments, but the amount of oxidation is slight and no oxidation as evidenced by changes in the sulfate sulfur occurred after subcutaneous injection. Brand and his associates (1) have reported excretions of extra sulfate

TABLE I

*Distribution of Extra Sulfur Excreted in Urine by Rabbit in 24 Hour Period Immediately Following Administration of S-Carboxymethylcysteine*

The cysteine derivative was administered in amounts equivalent to 400 mg. of sulfur. Extra sulfur values are expressed as per cent of the total sulfur administered. All experiments bearing the same letter were carried out with the same animal.

Experiment No.	Mode of administration	Extra sulfur		
		Total	Total sulfate	Organic
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A-1	Oral	94	11	83
A-3	"	93	17	76
A-2	Subcutaneous	77	0	77
A-4	"	76	1	75
B-1	Oral	65	-5	70
B-3	"	76	11	65
B-2	Subcutaneous	60	0	60
B-4	"	64	-7	71
C-3	Oral*	64	14	50 (26)†
C-4	Subcutaneous*	86	-3	89 (12)†
C-8	Oral			(17)†

\* In Experiments C-3 and C-4, the "cystine" contents as determined by the method of Shinohara and Padis (10) were 35 and 2 mg. respectively. In these experiments, the ether-soluble sulfur was also determined after treatment with zinc and hydrochloric acid. The absolute values of ether-soluble sulfur were 111 and 106, 55 and 53, and 73 and 73 mg. before and after reduction in Experiments C-3, C-4, and C-8 respectively.

† The values in parentheses indicate the percentage of the sulfur administered which was excreted as ether-soluble sulfur. This is presumably included in the organic sulfur fraction of the urine.

sulfur corresponding to 40 and 15 per cent respectively when the compound was fed to a normal and a cystinuric man. These results are calculated as the percentages of total extra sulfur excreted and not as the percentages of the sulfur fed as S-carboxymethylcysteine. Since the total extra sulfur excretion is not

given, it is not possible to compare these results directly with our own.

When S-carboxymethylcysteine was fed, a positive cyanide-nitroprusside test for disulfide linkages was readily obtained in the urine excreted, and, as shown in Experiment C-3 (Table I), there was present an appreciable amount of disulfide as determined by the method of Shinohara and Padis. S-Carboxymethylcysteine itself neither gave a positive cyanide-nitroprusside test nor reacted in the Shinohara-Padis method. When the cysteine derivative was injected subcutaneously, no positive cyanide-nitroprusside test was given by the urine nor was there an appreciable amount of disulfide present as determined quantitatively (Experiment C-4, Table I). Similarly in two experiments not recorded in Table I, no "extra" cystine (disulfide linkages) was present in the urine as determined by the method of Rimington after either oral or subcutaneous administration of S-carboxymethylcysteine.

Brand (1) reported that no ether-soluble sulfur was present in the urine excreted after the feeding of S-carboxymethylcysteine to men, but that after the urine was reduced with zinc and hydrochloric acid, an ether-soluble fraction was obtained which reacted positively with cyanide and nitroprusside and gave the color reaction described by Goddard and Michaelis (12) for thioglycolic acid. In our experiments (Nos. C-3, C-4, and C-8, Table I) extra ether-soluble sulfur was present in considerable amounts in the urine before reduction, and the amounts were not increased after reduction with zinc and hydrochloric acid. No thioglycolic acid test could be obtained. The urines excreted after feeding gave an ether extract which had a strongly positive reaction in the cyanide-nitroprusside test but no similar test could be obtained\* in the ether extracts of the urine after subcutaneous injection. The ether extract from urines after S-carboxymethylcysteine feeding also contained a substance which gave a cuprous mercaptide precipitate after reduction (13). It is thus evident that we have been unable to demonstrate the presence of thioglycolic acid or a complex yielding thioglycolic acid after reduction in the urine excreted after either oral or subcutaneous administration of S-carboxymethylcysteine to *rabbits*. Brand's hypothesis of the excretion of a mixed disulfide of thioglycolic acid and cysteine was based largely upon positive tests for thioglycolic acid in similar feeding

experiments with *man*. Our *feeding* experiments with rabbits indicated the presence in the urine of a disulfide linkage, but we were unable to show that thioglycolic acid was a component of the disulfide derivative. Since the sulfur of thioglycolic acid is readily oxidized to sulfate by the rabbit (14), it seemed improbable that if, as suggested by Brand, thioglycolic acid were split off from the molecule of S-carboxymethylcysteine, its sulfur would escape oxidation to any great extent.

In view of Stekol's finding that S-benzylcysteine was acetylated before excretion by the organism of various species (15), Tarver and Schmidt (16) have suggested that S-carboxymethylcysteine might similarly be excreted as the acetyl derivative. An attempt was made to demonstrate the presence of acetyl groups (17) in the ether extract of the urines. If acetylated derivatives of the sulfur-containing amino acids are present, the ratio of acetyl groups to sulfur of the ether extract should be 1.34. In order to test this procedure, a rabbit was fed 1 gm. of monobromobenzene, the 24 hour urine was collected, acidified, extracted with ether, and the extract was analyzed. The administration of monobromobenzene is known to lead to the excretion of bromophenylmercapturic acid, an acetylated derivative of cysteine. The ratio of acetyl groups to sulfur was 1.53. Since the experimental error of the Clark method is considerable, this was considered satisfactory and it was concluded that in this way acetylated sulfur-containing compounds could be detected, if present in significant amounts. When this procedure was applied to urine excreted by rabbits fed S-carboxymethylcysteine, the acetyl to sulfur ratio was approximately 0.092, indicating no more acetyl groups than are present in normal rabbit urine similarly extracted and within the limits of error of the procedure.

#### SUMMARY

1. Studies of the urinary partition of sulfur failed to show oxidation to sulfate of the sulfur of S-carboxymethylcysteine by the organism of the rabbit when the compound was injected subcutaneously. A slight increase in the sulfate sulfur of the urine was observed after oral administration, which is believed to be related to the activities of the intestinal microflora.

2. After oral administration of S-carboxymethylcysteine, a

substance soluble in ether, which gave a strongly positive cyanide-nitroprusside test for disulfide linkages, was excreted in the urine; no similar substance could be detected in the urine after subcutaneous injection of the cysteine derivative.

3. It was not possible to demonstrate that S-carboxymethylcysteine was acetylated to form an N-acetyl compound similar to the mercapturic acids.

4. When S-carboxymethylcysteine was administered orally or subcutaneously to *rabbits*, no evidence of the excretion in the urine of a disulfide of whose molecule thioglycolic acid was a component part, as postulated by Brand (1) in similar experiments with *man*, was obtained.

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## THE METABOLISM OF SULFUR

### XXX. THIOUREA

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The recent report of Medes (1) who in experiments on man observed an increase in conjugated sulfate sulfur and in disulfide compounds excreted after the oral administration of thiourea has led us to investigate anew the fate of this substance in the organism of the rabbit. Earlier unpublished studies of one of us (L.) had suggested that the administration of thiourea to rabbits resulted in slightly decreased oxidized (sulfate) sulfur excretions in the urine. Since, according to Medes, the disulfide compound excreted was largely cystine (the compound reacting in the specific naphthoquinonesulfonic acid reaction of Sullivan), it seemed possible that the decreased excretion of sulfate sulfur might be explained by a failure of oxidation and the excretion of sulfur as cystine or a complex derived from it, which would under normal conditions have appeared in the urine as sulfate sulfur. Medes suggested that a complex of cysteine and thiourea might be formed and that, after excretion in the urine, this complex might hydrolyze to thiourea and cysteinesulfenic acid, which in turn in *in vitro* experiments has been believed to undergo dismutation to yield cysteine and cysteinesulfonic acid (2).

The experimental methods and procedures were those commonly used in experimental studies of sulfur metabolism in this laboratory (3). Commercially available thiourea of chemically pure grade was recrystallized from alcohol and the recrystallized material was fed or injected. It melted at 178–179° (corrected) and contained 41.79 per cent of sulfur and 36.55 per cent of nitrogen (theoretical, 42.02 and 36.81 per cent respectively). The purity of the *l*-cystine and of *dl*-methionine was assured by analyses and, in the case of the cystine, by measurement of the specific rotation.

A condensed protocol of a typical experiment is shown in Table I. Both total and organic sulfur were considerably increased. It is to be noted, however, that the extra total sulfur is significantly less than the extra organic sulfur. While the extra organic sulfur accounted for 86 and 78 per cent of the sulfur of the thiourea administered in Experiments B-13 and B-14 respectively, the extra total sulfur values were only 65 and 63 per cent of the thiourea sulfur. This curious difference is explained by the decreased values of the oxidized (sulfate) fraction. After feeding thiourea

TABLE I

*Typical Experiment Demonstrating the Effect of Administration of Thiourea on Sulfur Partition of Urine of Rabbit*

Periods 1, 3, and 5 are the averages of normal excretions of 4 to 5 days. The experimental periods consisted of 2 days on each of which thiourea equivalent to 200 mg. of sulfur was administered. The uniform daily diet of cabbage was eaten completely each day, the experimental days included. All results are average *daily* excretions. Rabbit B, 3.75 kilos.

Period No.	Experiment No.	Urea nitrogen (a)	Sulfur					(a) (b)
			Total	Sulfate			Organic	
				Total	Inorganic (b)	Conjugated		
		mg.	mg.	mg.	mg.	mg.	mg.	
1	B-13*	808	149	101	96	5	48	8.4
2		778	278	60	54	6	218	14.4
3		922	150	107	100	7	43	9.2
4	B-14†	750	274	71	66	5	204	11.4
5		788	170	110	105	5	60	7.5

\* Oral administration.

† Subcutaneous injection.

(Experiment B-13), the decrease in total sulfate sulfur was 41 per cent of the average control value and after injection (Experiment B-14), 30 per cent. It was possible that the lower values for oxidized sulfur were due to a depression of the general level of protein metabolism. That this explanation is not correct is shown by the excretion of urea nitrogen and the ratios of urea nitrogen to sulfate sulfur. There was noted some decrease in the urea of the urine, but the decrease in sulfate sulfur did not run parallel to the lowered urea excretion. The ratios of urea nitrogen to

inorganic sulfate sulfur ((a)/(b) in Table I) increased from an average value in the control periods of 8.4 to 14.4 and 11.4 respectively. If the "expected" excretion of sulfate sulfur is calculated by dividing the urea excreted in the experimental periods by the normal ratio, the decreases in total sulfate sulfur of the urine actually observed were 33 and 18 per cent of the expected excretion in the two experiments. It appears from this and similar calcula-

TABLE II

*Distribution of Extra Sulfur and Decreased Oxidized Sulfur Excretion in the Urine of Well Fed Rabbits After the Administration of Thiourea*

In Experiments B-13, B-14, and D-4, thiourea equivalent to 200 mg. of sulfur was administered daily on each of 2 successive days; in other experiments the equivalent of 400 mg. of sulfur was given on a single day.

Extra sulfur is expressed as percentage of the total sulfur administered and decrease in total sulfate sulfur is calculated from the average daily excretion of sulfate sulfur of the control periods (a) and from the expected excretion as calculated from the ratio of normal urea nitrogen to total sulfate sulfur (b) as described in the text. Experiments bearing the same letter were carried out with the same animal.

Experiment No.	Administration	Extra sulfur		Decrease in total sulfate sulfur				Ether-soluble sulfur	
		Total	Organic	(a)		(b)			
		per cent	per cent	mg.	per cent	mg.	per cent	mg.	per cent
B-7	Oral	68	83	66	40	48	33	270	67
B-10	"	47	78	124	75	116	74	215	54
B-13	"	65	86	42	41	30	33	77	39
B-8	Subcutaneous	50	71	84	51	64	44	222	56
B-9	"	50	75	110	67	84	60	231	58
B-14	"	63	78	31	30	15	18	45	23
D-4	"	33	75	83	64	61	56	103	52

tions of Table II that thiourea acted specifically in decreasing the excretion of oxidized sulfur.

The points just discussed are further emphasized by the data of Table II in which are summarized seven of the fourteen experiments we have carried out with well fed animals. In some of the experiments with thiourea, a moderate toxicity was noted, manifested chiefly by a slight loss of appetite and a temporary failure to eat all of the rabbit chow offered; however, the cabbage was always consumed. The question arose as to whether the de-



creased sulfate sulfur excretion was related to the lessened food consumption. In order to check this, the animals were fed cabbage only, which was always consumed completely on the experimental days. In these experiments, three of which (Experiments B-13, B-14, and D-4) are summarized in Table II, the decreased excretion of oxidized sulfur was always observed. In the other experiments of Table II, a small part of the chow was refused, but the results are not significantly different.

Experiments, as yet unpublished, have shown that, after short fasts, the oxidized sulfur of the urine of the rabbit is decreased to a greater extent than is the urea nitrogen, as shown by the fact that in such experiments, the urea nitrogen to sulfate sulfur ratio is greatly increased, which suggests that under these conditions in this species, sulfur is retained to a greater extent than is the nitrogen. The diminished sulfate sulfur excretions resemble those observed after the administration of thiourea as already discussed. When thiourea is fed to or injected into a fasting animal, the effects on the sulfur excretion seem to be additive in part; that is, the decreased sulfate sulfur excretion is more marked when thiourea is fed to a fasting rabbit than when the animal is fasted and no thiourea is given. The protocol of a typical experiment of a series of seven such experiments is recorded in Table III.

Slight increases in the conjugated sulfate sulfur after the administration of thiourea to rabbits (4, 5) and to man (1) have been reported. In none of the twenty-one experiments of the present series in which thiourea has been fed to or injected into well nourished or fasting rabbits have we observed any increases of significance in this sulfur fraction of the urine. It must be remembered that these values are obtained by difference and that slight variations must be interpreted with caution. The data of Table I are typical of those obtained in all our experiments.

The presence of thiourea in the urine after its administration could be demonstrated qualitatively (6). As an index of the quantitative relationships the ether-soluble fraction of the urinary sulfur was determined (7). Thiourea is decomposed in weakly acid and alkaline solution to hydrogen sulfide or alkali sulfides, carbon dioxide, and an ammonium salt; in higher concentrations of alkali, there are formed ammonia, thiocyanate, and sulfides (8). In order to avoid such decomposition, extractions were carried

out in neutral reaction, but recovery of known concentrations of thiourea was never greater than 85 per cent and considerable variations were observed between aliquots of the same solution. The results (Table II) must therefore be considered as minimal and as approximate estimations of the ether-soluble sulfur only. However, a very considerable part of the organic sulfur of the experimental days was included in the ether-soluble fraction as determined. In a further study, the 24 hour urine collected after

TABLE III

*Effect of Inanition with and without Simultaneous Oral Administration of Thiourea on Excretion of Urinary Sulfate in Rabbit*

During the control periods of 4 to 6 days each, the rabbit was fed a constant amount of chow and cabbage. After a 2 day fast, the animal again received the usual diet. During the thiourea period the animal was fasted and received orally each day thiourea equivalent to 200 mg. of sulfur. This was followed by a third feeding control period. For the calculation of the expected excretion the text should be consulted. Rabbit D, 3.15 kilos.

Period	Urea nitrogen (a)	Total sulfate sulfur				(a) (b)
		Ex- creted (b)	Ex- pected (c)	(c) - (b)		
	mg.	mg.	mg.	mg.	per cent of (c)	
Control Period A.....	1818	144				12.5*
Fast, 1st day.....	1272	64	112	48	43	19.9
“ 2nd “ .....	906	35	79	44	55	25.9
Control Period B....	1631	145				11.2*
Thiourea, 1st day.....	1043	32	91	59	65	32.6
“ 2nd “ .....	920	27	80	53	66	34.0
Control Period C.....	1659	160				10.4*

\* Average ratio, 11.4.

1 gm. of thiourea was fed was extracted with ether in a continuous extraction apparatus for 16 hours and the ether was removed from the extract by evaporation. After the residue was dried over phosphorus pentoxide, about 300 mg. of material were obtained which was recrystallized several times from absolute alcohol. The crystals gave the Grote test (6), a precipitate with mercuric chloride, and, when heated, the ferric chloride test for thiocyanate. The melting point was the same as that of pure thiourea, and when

the substance was mixed with crystals of pure thiourea, the melting point did not change. Analyses of two different products thus isolated gave 41.41 and 41.00 per cent of sulfur (theoretical, 42.02 per cent). This demonstrates that a considerable portion at least of the thiourea must be excreted in the urine as such or as an easily dissociated derivative.

The only indication of an increase in the disulfide fraction of the urine after administration of thiourea, as suggested by Medes (1), was a pink color which developed when the urine was allowed to stand several hours in the cyanide-nitroprusside test. Thiourea will give this test owing to its decomposition in alkaline solution, as pointed out by Pavolini (9). A positive cystine test could not be obtained in the urine with either the Sullivan test or the Prunty modification in which the cystine is first reduced with zinc and hydrochloric acid (10). A precipitate could be obtained when the urine excreted after thiourea was treated with cuprous chloride as described by Medes and Padis (11), but this precipitate contained no substances which gave a clearly positive cystine test. Thiourea is precipitated by cuprous chloride under these conditions, so that the precipitate was probably the cuprous mercaptide of thiourea.

If a disulfide may be formed by the rabbit from thiourea and cysteine, as has been suggested by Medes in experiments on man (1), the presence of cystine or methionine in excess in the organism might be expected to favor the formation of such a compound. Such a reaction might be evidenced by a failure to oxidize the cystine used for complex formation, which would result in a decreased recovery of the sulfur of cystine as oxidized sulfur in the urine when cystine was fed with thiourea. Cystine and methionine were accordingly fed to a rabbit and the recovery of extra sulfur in the urine was determined. The experiments were then repeated with the additional administration of thiourea (Table IV). Extra sulfur is calculated as the amount in excess of the normal control excretions. Since the sulfur of thiourea is not oxidized to sulfate by the rabbit, the values presented indicate that cystine was oxidized and excreted as sulfate to about the same extent when thiourea was present in the body as in its absence. Since we believe that thiourea depresses the normal excretion of sulfate sulfur, if this is true when cystine and thiourea are fed

together, the results of the present experiment could be interpreted as an indication of a more complete degree of oxidation of the sulfur of cystine when fed with thiourea than when fed alone. In any event, there is no indication of a decreased oxidation of the sulfur of cystine due to the presence of thiourea. We hesitate to attempt an interpretation of the results of the single experiment with methionine in view of the variability of the recovery of oxidized sulfur after methionine feeding to rabbits previously observed in our laboratory (3). The apparent decreased recovery of oxidized sulfur when methionine and thiourea are fed together might be explained, if the contention of Medes is correct, on the theory that cysteine formed from methionine has a different metabolic history

TABLE IV

*Recovery and Partition of Extra Urinary Sulfur after Feeding Cystine or Methionine Alone and with Thiourea*

Rabbit B, weight 4 kilos.

Sulfur fed as			Extra sulfur as		
Cystine	Methionine	Thiourea	Total	Total sulfate	Organic
mg.	mg.	mg.	mg.	mg.	mg.
200	0	0	106	85	21
200	0	200	243	83	160 (111)*
0	200	0	107	94	13
0	200	200	250	58	192 (52)*

\* The values in parentheses represent ether-soluble sulfur.

than orally administered cystine has and that this cysteine or an intermediary product might react with thiourea. A difference in the metabolic behavior of cystine and cysteine is suggested by the studies of the fate of these amino acids when administered to cystinurics (12). We are not warranted in suggesting such an interpretation on the basis of a single experiment and incomplete data, but since it is not possible to continue this investigation in the near future, the results are presented.

Two other possible products of the metabolism of thiourea suggest themselves, thiocyanate and thiosulfate.<sup>1</sup> Tests of the

<sup>1</sup> A study of thiosulfate excretion in the urine after the administration of various sulfur-containing compounds by Dr. J. H. Gast and one of the authors (L.) will be reported shortly.

experimental urines did not show any significant increases in these ions after the administration of thiourea.

#### SUMMARY

1. After oral or parenteral administration of thiourea to rabbits, there was no evidence from the study of the partition of the urinary sulfur that the sulfur portion of the molecule was oxidized to sulfate. The greater part of the thiourea appeared to be excreted as such in the urine, as demonstrated by isolation as well as by increases in the ether-soluble sulfur fraction.

2. The presence of small and increased amounts of cystine in the urine after the administration of thiourea as observed in *man* by Medes (1) could not be demonstrated in the rabbit.

3. The administration of thiourea resulted in a decrease in the oxidized (sulfate) sulfur but no increase in conjugated sulfate sulfur of the urine. This decrease in oxidized sulfur is not believed to be due to a general effect on protein metabolism, since the urea nitrogen was not decreased proportionally as shown by changes in the urea nitrogen to sulfate sulfur ratio. Short periods of inanition in rabbits also led to a decreased sulfate sulfur excretion without a corresponding decrease in urea excretion; the oxidized sulfur excretion of the fasted animal was decreased still further by the administration of thiourea.

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# INVESTIGATIONS IN ENZYMATIC HISTOCHEMISTRY

## III. DISTRIBUTION OF ENZYMES IN RABBIT KIDNEY

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The arginase activity of rabbit kidney was shown, in a previous paper (1), to be specifically associated with the cells of the proximal convoluted tubules. This investigation in enzymatic distribution has now been extended to cathepsin, aminopolypeptidase, dipeptidase, esterase, and amylase. The results of this study demonstrate that histological variations within the structure of the kidney are correlated with variations in the content of certain enzymes.

In the past, investigations concerning the functions of various tubule cells in the kidney were conducted primarily from the physicochemical point of view; it is hoped, however, that an enzymatic approach to this problem may contribute to a better understanding of the subject.

### EXPERIMENTAL

Before the actual histoenzymatic work could be undertaken, it was necessary to make a study of those kidney enzymes which were to be investigated. Since the literature yielded very little information concerning the proteinase of kidney, preliminary research into the character of this enzyme was necessary.

The pH optimum of kidney proteinase was measured; clupein and edestin were used as substrates. In the case of clupein sulfate, the progress of the enzymatic breakdown was followed by micro formol titration (2), while in the case of edestin, micro acetone titration (3) was used. Minced rabbit kidney was extracted for 24 hours with 10 volumes of 60 per cent glycerol. The filtrate, diluted 1:6 with water, was used for the enzymatic

determinations. To 7 c.mm. of kidney extract were added 7 c.mm. of water or of cysteine solution (10 mg. in 1 cc.) at the proper pH, 7 c.mm. of veronal-HCl buffer (4), and 7 c.mm. of 5 per cent clupein sulfate or 5 per cent edestin solution at the required pH. The rate of decomposition was measured after 4 hours incubation at 37°, as described in a previous paper (5). The results obtained have been presented in Table I. In the same table have been shown the results of studies on kidney

TABLE I

*pH Optima of Kidney Cathepsin, Dipeptidase, and Aminopolyptidase*

The activity is expressed in c.mm. of 0.05 N HCl.

Substrate	pH											Enzyme
	3.0	3.5	4.0	4.4	5.0	6.0	6.5	7.0	7.5	8.0	8.6	
Clupein sulfate	0.62		1.56		2.48	2.06		1.88		0.42		Cathepsin
Clupein sulfate + cysteine	1.06		2.28		5.46	5.04		4.66		2.66		"
Edestin*		1.62	2.30	2.12	1.98							"
" + cysteine		4.06	5.46	5.40	4.96							"
Glycylglycine						1.96	2.20	2.48	2.62	2.50	2.32	Dipeptidase
dl-Leucyl-diglycine						2.56	2.88	3.00	3.20	3.06	2.68	Aminopolyptidase

\* Edestin precipitates at higher pH and therefore only the acid side could be investigated.

dipeptidase and aminopolyptidase contained in the same extract. 7 c.mm. of 0.2 M glycylglycine or 7 c.mm. of 0.2 M dl-leucyldiglycine were added to 7 c.mm. of kidney extract, and after 1 hour's incubation at 25° the increase of amino groups was measured by micro acetone titration (3). The results are reported in c.mm. of 0.05 N HCl.

As indicated in Table I, the pH optimum for clupein sulfate decomposition was at 5, while the optimum for edestin was found

to be at pH 4. In both cases the addition of cysteine produced a marked activation, indicating that the enzyme responsible for the protein decomposition is of the catheptic type. Both the dipeptidase and the aminopolypeptidase of the kidney had pH optima at 7.5.

Determinations were also made of the decomposition rate of the above substrates under the action of the enzymes. By confining our experiments so that the values obtained were within the linear portion of the curve, these values could be used as a measure of the enzyme concentration.

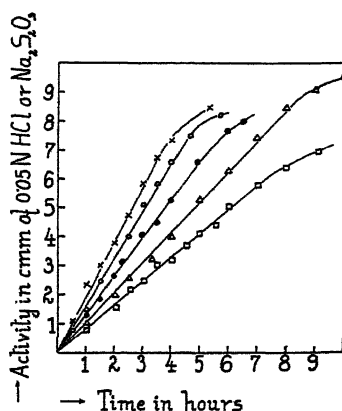


FIG. 1. The rates of enzymatic decompositions of various substrates.  $\times$  dipeptidase activities,  $\circ$  aminopolypeptidase activities,  $\bullet$  esterase activities,  $\Delta$  cathepsin activities,  $\square$  amylase activities.

The results obtained with kidney esterase and amylase have been included in Fig. 1. The following procedure was used in making these determinations. 7 c.mm. of a solution containing 2 per cent Lintner starch, 1 per cent NaCl, and 0.1 M phosphate buffer at pH 6.8 were added to 7 c.mm. of kidney extract. After each incubation period at 37° the increase of glucose was measured by the method of Linderstrøm-Lang and Holter (6). For the kidney esterase determination, the method of Glick (7) was used. 7 c.mm. of kidney extract were employed, to which were added 7 c.mm. of methyl butyrate emulsion which had been prepared according to the method of Glick and then treated in the supersonic



oscillator to obtain a stable emulsion. Because of the high activity of kidney esterase the incubation temperature was lowered to 25°.

The preparation of rabbit kidney for histoenzymatic investigation was carried out in the same manner as that described in a previous paper (1). After saline perfusion, a tissue column was cut parallel to the flat side of the kidney; a borer of 2.5 mm. diameter was passed by means of a drill-press first through the pelvis, then the medulla, and finally the cortex. In this way a column was obtained containing all the structural elements of the kidney. The freezing-microtome and the histological technique were the same as were previously employed (8). Unfortunately, the freezing-microtome technique of Linderstrøm-Lang and Morgensen (9) could not be used in this work because of the damaging effect of freezing upon the cells of the kidney. The frozen tissue slices proved unsuitable for quantitative histological work.

For the histoenzymatic estimation of kidney cathepsin two adjacent slices 15  $\mu$  in thickness were placed in a micro test-tube charged with 7 c.mm. of water. The catheptic activity of the slices was estimated after a 4 hour incubation period as described above, with edestin as the substrate. Only the full activity (with cysteine) was measured.

In the histoenzymatic estimation of dipeptidase and aminopolypeptidase only one 15  $\mu$  slice was placed in the tube containing 7 c.mm. of water, and the determination carried out as described. Owing to the high activity of these two enzymes in the rabbit kidney it was necessary to reduce the incubation to 1 hour at 25°. For the estimation of esterase one 15  $\mu$  slice was used under the conditions described for the peptidase. Kidney amylase determination was made on a 20  $\mu$  slice by the technique described above with the exception that an incubation period of 20 hours at 37° was employed.

#### *Enzymatic and Cellular Distribution in Rabbit Kidney*

In order to ascertain which types of cells in the rabbit kidney were responsible for the catheptic and aminopolypeptidase activities a correlation between the enzymatic activity and the number of various cells had to be demonstrated. Parallelism between the enzyme curve and the curve representing cellular

counts should serve as evidence of such relationship. The technique of making cellular counts was that used by Linderstrøm-Lang, Holter, and Ohlsen (8) and Weil and Ely (1).

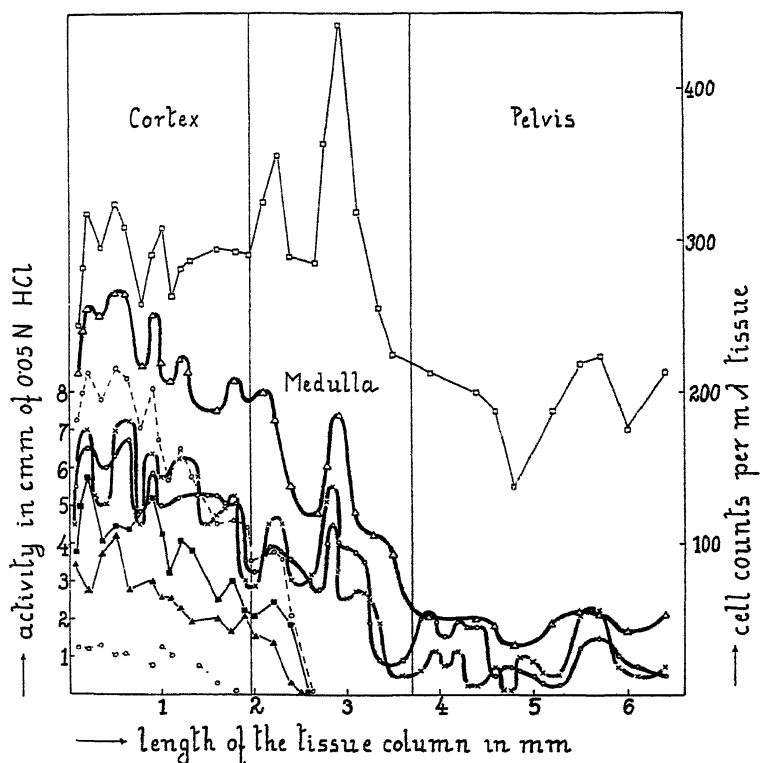


FIG. 2. The distributions of cathepsin and aminopolypeptidase activities and of various cells in rabbit kidney. ○—— cathepsin activities, × aminopolypeptidase activities, ■ cells of the proximal tubules, ▲ cells of the distal tubules, ○ ... relative areas of glomeruli, ○—— total numbers of cells of proximal and distal tubules, □ total number of cells, △ corrected total number of cells.

An effort was made to correlate the catheptic and aminopolypeptidase activities with the number of individual cells of the rabbit kidney. Figs. 2 and 3 show that such a correlation was not obtained, although the curves obtained with cathepsin and aminopolypeptidase run parallel, indicating that the two enzymes

are similarly distributed throughout the organ. The fact that both enzymes could be detected along the entire length of the tissue column indicated that they were not associated with a single type of kidney cell alone, and that it was desirable therefore

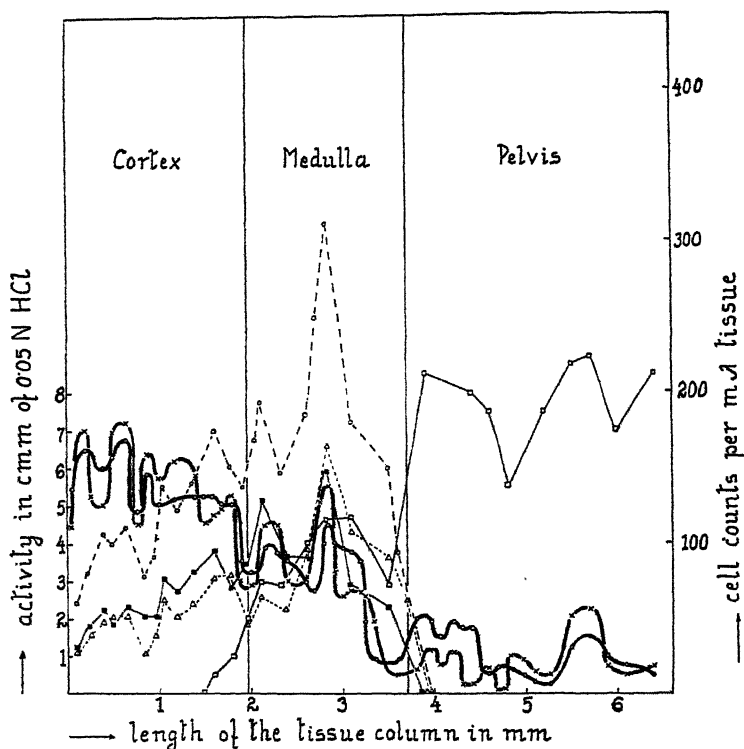


FIG. 3. The distributions of cathepsin and aminopolypeptidase activities and of various cells in the rabbit kidney.  $\circ$ — cathepsin activities,  $\times$  aminopolypeptidase activities,  $\blacksquare$  cells of the tubules of the descending Henle's loop,  $\circ$ — total number of cells of tubules of the descending and ascending Henle's loop,  $\square$  cells of the collecting tubules,  $\triangle$  cells of tubules of the ascending Henle's loop.

to take into account all possible variations of the cellular counts. The cortical part of the kidney, as Fig. 2 indicates, consists chiefly of the proximal and distal tubules, and since no relation appeared to exist between the enzyme activities and the number of cells of

either type alone, the possibility that both types of cells are responsible for the enzymatic activities in the renal cortex was considered. Graphic comparison of the enzymatic activities with the total number of cells from proximal and distal tubules combined (Fig. 2) strongly supports this possibility.

The medullary portion of the rabbit kidney, consisting chiefly of the cells of the ascending and descending tubules of Henle's loop as indicated in Fig. 3, showed a marked catheptic and aminopolypeptidase activity, though less than that of the cortex. The enzymatic activities again compared graphically with the total cell count, this time of the ascending and descending tubules of Henle's loop, showed a definite relationship.

In the pelvic portion of the kidney tissue column the histological structure is very simple, consisting, as indicated in Fig. 3, only of cells of the collecting tubules. Since catheptic and aminopolypeptidase activities were demonstrable in this portion, such enzymes must be present in cells of the collecting tubules although the concentration of these enzymes was much lower than in the cortex or medulla.

In view of both enzymatic activities in all structural elements of the rabbit kidney, a parallel between the graphical representation of the total number of cells at given levels and the enzymatic activity at corresponding points might have been expected. The parallelism so obtained, however, is of a qualitative character in that the elevations and depressions of the enzymatic curves parallel the elevations and depressions of the curve of the cell count only in the sense of being similarly located, while differing in height. Of course, entire parallelism could be expected only if all the various cells were presumed to possess the same enzyme concentration. Dividing the enzymatic activity at a given level by the corresponding total number of cells provided an easy means of estimating the enzyme concentration of a single cell at that point. When this was done, it was found that the cells of the cortical region possessed about twice the enzymatic activity of the medullary cells, and about 4 times that of the cells in the pelvis, which is to say the cells of the collecting tubules. Since the cortex consisted primarily of proximal and distal tubules, the medulla of ascending and descending tubules of Henle's loop, and the pelvis of collecting tubules, it could be

assumed that one cell of a convoluted tubule (proximal or distal) was the enzymatic equivalent of two cells from Henle's loop and of four cells from the collecting tubule. Having made this assumption, the graphical representation of the cell count was corrected as follows: the total number of proximal and distal convoluted tubule cells was recorded as found, the number of cells of Henle's loop was divided by 2, and the number of cells of collecting tubules divided by 4. The curve of the corrected total number of cells not only retained the qualitative parallelism with the enzymatic curve, but showed in addition a definite quantitative correlation. The results, therefore, appeared to justify the assumptions made concerning the relative enzymatic content of the various types of cells.

The conclusions drawn from this experiment were that the catheptic and aminopolypeptidase activities might be found in all the cells of the rabbit kidney, while the quantitative distribution varied with the type of cell. The cells of the convoluted tubules were capable of about twice the activity of the cells of the loop of Henle, in the case of cathepsin and aminopolypeptidase, and about 4 times that of the cells of the collecting tubules.

Owing to the high enzymatic activity of the cells of the proximal and distal convoluted tubules, the enzyme content of the glomeruli could not be estimated. To decide this question it would be necessary to isolate pure glomerular tissue.

A second experiment carried out in the same manner confirmed this conclusion; the results have been presented in Fig. 4. For the sake of clarity Fig. 4 shows only the curve for cathepsin together with a curve representing the corrected total number of cells as in Fig. 2. The correlation so obtained again appeared to be satisfactory.

The location of the catheptic and aminopolypeptidase activities in the rabbit kidney having been established, the catheptic activity was used as a guide for the localization of dipeptidase, esterase, and amylase activities in the subsequent experiments. This method was less time-consuming than that involving the cell counts and proved to be very helpful. In order to localize each enzyme a simultaneous determination of catheptic activity was made at each chosen level in the tissue column. Fig. 5 indicates the histoenzymatic distribution of dipeptidase and

catheptic activities. The curves were found to be definitely parallel in the cortical and medullary regions, while the pelvis was practically free of dipeptidase activity under the experimental

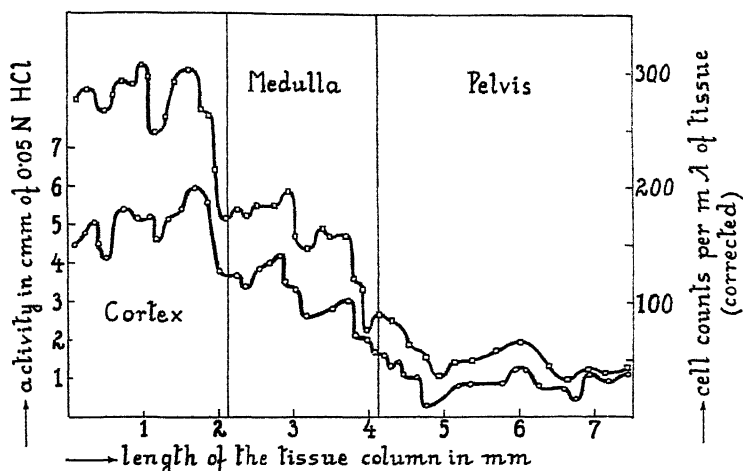


FIG. 4. The correlation of cathepsin activities and the corrected total number of cells in the rabbit kidney. ○ cathepsin activities, □ corrected total number of cells.

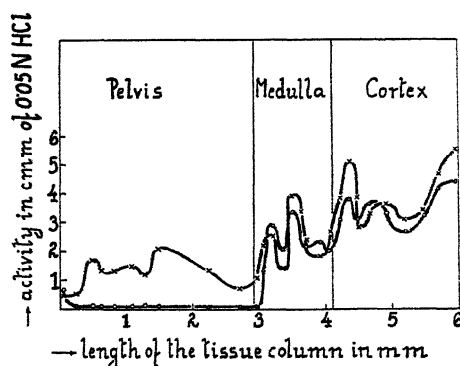


FIG. 5. Histochemical distributions of cathepsin and dipeptidase activities in rabbit kidney. × cathepsin activities, ○ dipeptidase activities.

conditions used. It was concluded that the distribution of kidney dipeptidase was the same as that of cathepsin and aminopolypeptidase in the cortical and medullary region of the rabbit

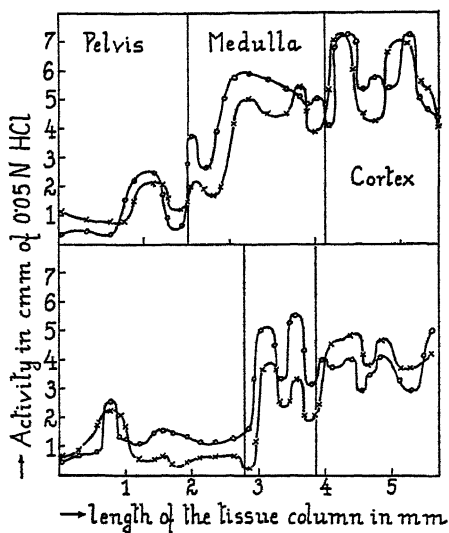


FIG. 6. Histochemical distributions of cathepsin and esterase activities in rabbit kidney.  $\circ$  cathepsin activities,  $\times$  esterase activities.

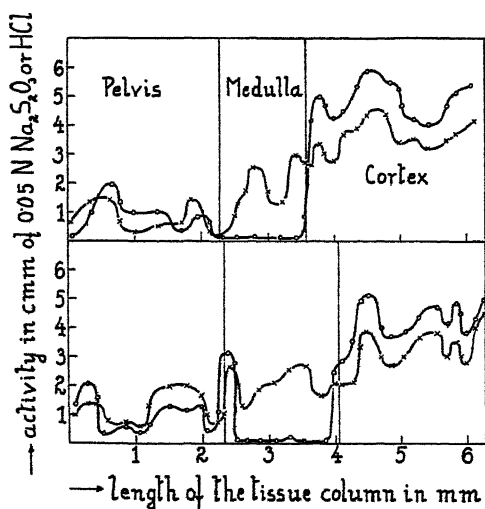


FIG. 7. Histochemical distributions of cathepsin and amylase activities in rabbit kidney.  $\times$  cathepsin activities,  $\circ$  amylase activities.

kidney, but was absent, or present only in very low concentration, in the pelvis and therefore in the cells of the collecting tubules.

In Fig. 6 the activities of kidney esterase have been compared with the catheptic activities in the same way that the dipeptidase was compared in the previous experiments. A marked parallelism throughout the entire length of the tissue column strongly supported the conclusion that the histoenzymatic distribution of kidney esterase was the same as that indicated for cathepsin and aminopolypeptidase in Figs. 2 and 3.

Experiments carried out in the same way on kidney amylase led to a different picture, as shown by Fig. 7. The amylase activities followed closely the catheptic activities in the cortical region of the rabbit kidney. As soon as the medullary portion of the kidney was reached, the amylase activity disappeared, reappearing again in the pelvis where it again paralleled the catheptic activities. The conclusion appeared justified that the source of amylase activity in the cortex was the same as that of catheptic activity, while the cells of Henle's loop (constituting the bulk of the medullary portion of the kidney) were practically free from amylase activity<sup>1</sup> although possessing considerable catheptic activity. In the pelvic region, and therefore in the collecting tubules, the catheptic and amylase activities were again parallel.

#### SUMMARY

Histoenzymatic investigations have shown that catheptic, aminopolypeptidase, and esterase activities may be demonstrated in all structural elements of the rabbit kidney. The quantitative distribution of these enzymes, however, varied among the different types of kidney cells; that is, the cells of the proximal and distal convoluted tubules were about twice as active enzymatically as the cells of the ascending and descending tubules of Henle's loop and about 4 times as active as cells of the collecting tubules.

<sup>1</sup> It should be mentioned that some of the cells of the collecting tubules were present in the medullary portion of the kidney (see Fig. 3), and it was therefore surprising that their presence did not demonstrably influence the amylase activity in this region, since the cells of these tubules did exhibit this activity in the pelvic region although to a lesser degree than do the cells of the cortex.



The same distribution was found for dipeptidase activities with the exception that cells of the collecting tubules did not possess any of this activity under the conditions of the experiment. Amylase activity in the rabbit kidney was found to be associated with the cells of the proximal and distal convoluted tubules and with the cells of the collecting tubules. The cells of the loops of Henle were free from amylase activity.

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## RADIOACTIVE IODINE AS AN INDICATOR OF THE METABOLISM OF IODINE

### I. THE TURNOVER OF IODINE IN THE TISSUES OF THE NORMAL ANIMAL, WITH PARTICULAR REFERENCE TO THE THYROID\*

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The distribution of iodine in various tissues in both normal and abnormal states of thyroid activity has not lacked considerable study, but, as Salter has pointed out (1), these investigations have suffered from a lack of analytical procedures suitable for the accurate determination of the minute amounts normally present in most tissues. The approach to the problem of iodine metabolism in certain of its phases can be simplified by the use of radioiodine.<sup>1</sup> In general, two types of problems lend themselves to study with this new tool: (a) *The fate of exogenous iodine.* Various amounts of administered iodine can be studied in and out of tissues much more accurately by means of radioactive measurements than by accompanying changes in concentration. Furthermore, when only the radioactivity is being measured, the amount of iodine administered can be reduced to any desired level without loss of accuracy in the analysis. (b) *The turnover of endogenous iodine.* It is possible to label the circulating iodine within the animal without altering the amount of iodine already present, since strong

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<sup>1</sup> The sensitivity of procedures involving radioactive measurements has been discussed elsewhere (2).

samples of radioiodine can be prepared practically free of iodine.<sup>2</sup> By this means the *normal* turnover of circulating iodine can be observed. This feature can be obtained at present only by a labeling device as sensitive as that provided by the radioactive isotope.

Previous work on radioactive iodine has dealt, for the most part, with its accumulation in the thyroid gland (4-8). In the present investigation the turnover of administered labeled iodine was measured in the liver, muscle, brain, blood, small intestine, kidney, testes, and adrenals, as well as in the thyroid gland. Observations on the rate of its absorption and excretion are also recorded.

#### EXPERIMENTAL

Radioactive iodine was prepared by bombardment of tellurium with deuterons (3). The tellurium was scraped from the target and placed in a distilling flask. Nitric acid was added and the iodine distilled into carbon tetrachloride. After the carbon tetrachloride was washed free of nitric oxide with water, most of the iodine was removed by shaking with a dilute solution of  $\text{Na}_2\text{S}_2\text{O}_3$ . The sample of radioactive iodine so obtained contains only an insignificant amount of iodine. The number of radioactive atoms in the sample is far below the minimum quantity necessary for chemical detection and is even far less than the minute amounts of ordinary iodine inevitably present in the tellurium, reagents, vessels, etc. The sample is here referred to as the dose without carrier or the "tracer" dose.<sup>3</sup>

A considerable amount of radioactive iodine fails to come over in the distillation. A small amount of KI may be added as carrier to the tellurium residue and the distillation repeated. Such samples, containing measurable amounts of iodine as carrier, may be used when the distribution of exogenous iodine is to be observed.

Two types of iodine administrations were used: (a) *Tracer doses*, for which the iodine sample without carrier obtained in the first

<sup>2</sup> This radioiodine is prepared by bombarding tellurium with deuterons (3). When iodine is separated, it contains the radioactive atoms plus the unavoidable traces of iodine present in reagents, glassware, and atmosphere. Neither source of iodine is measurable by chemical analysis.

<sup>3</sup> We are indebted to Dr. J. G. Hamilton for the isolation of all samples of radioiodine used in the present investigation.

distillation was used. As already pointed out, this sample contains practically no iodine, and its administration serves to label mainly the *circulating* iodine without altering its concentration. In the experiments to be described below, it was fed by stomach tube as an aqueous solution or injected subcutaneously as an isotonic NaCl solution. (b) *Relatively large amounts of iodine*, for which the labeled iodine obtained with KI carrier was used.

### *Isolation and Determination of Radioactive Iodine in Tissues*

The method described below was designed primarily to facilitate the measurement of radioiodine in a tissue without regard to its total iodine content. Hence in contrast to the usual methods for the determination of iodine there is no lower limit of iodine concentration in a tissue to which the measurement of radioiodine cannot be applied *so long as an adequate amount of radioactivity is present*. With reasonable care a quantitative yield of iodine (as judged by recovery of radioactivity) can be obtained from minute quantities as well as from larger amounts of tissue. Since only radioactivity was being measured, iodide was introduced as carrier wherever it was found convenient. In all of the tissues analyzed in the present investigation, inactive carrier in the form of KI was added. It is worthy of note that the determination of radioiodine is much simpler than the usual methods for the estimation of total iodine in that the reagents employed need not be free of small amounts of iodine or other substances that may have an apparent iodine equivalent. The only condition in the use of reagents for the determination of the radioiodine content of a tissue is that they shall cause no loss of iodine or interfere with its quantitative extraction by the solvents employed.

The method involves the oxidation of all the iodine in the tissue to iodate by ashing with chromic acid. This is followed by reduction of the excess chromate to the chromic state and of the iodate to iodide by means of  $\text{SO}_2$ . After the excess  $\text{SO}_2$  is boiled off, an excess of iodate is added and the resulting iodine extracted with carbon tetrachloride. In order to mount the sample the iodine is reduced with  $\text{Na}_2\text{S}_2\text{O}_3$  and precipitated on hardened filter paper as AgI. Eight or more samples of tissue can be conveniently run at the same time. With the vessels described below, amounts of tissue up to 15 gm. can be conveniently handled.

*Procedure*

The tissues were transferred to 300 cc. Kjeldahl flasks and 1 cc. of 0.02 M KI solution<sup>4</sup> added as a carrier. The ashing was carried out with chromic acid; for each gm. of material, 4 cc. of 50 per cent chromium trioxide and 8 cc. of 1:1 H<sub>2</sub>SO<sub>4</sub> were found best. The flasks were heated for 1.5 to 2.5 hours, depending on the quantity of tissue. Water was added from time to time when the volume became reduced to the point where substances began to crystallize out. It is important to ensure complete oxidation, since unashed material hinders the subsequent extraction of iodine. Completeness of ashing can be readily gaged by the absence of all particles and of the odor characteristic of partially ashed material.

The contents of the flasks were then diluted to 150 to 200 cc. with water and SO<sub>2</sub> passed through in series; the outlet was run into a bottle of NaOH. The flasks were immersed in cold water during this reduction process, since considerable heat was produced. Eight samples were usually treated at the same time and reduction considered complete when vigorous rotation of the last flask in series failed to suck back NaOH from the absorption bottle while SO<sub>2</sub> was being bubbled through slowly.

The Kjeldahl flasks were then heated to drive off excess SO<sub>2</sub>. To avoid possible oxidation and loss of iodide, it is advisable to stop heating immediately after the last of the SO<sub>2</sub> is driven off. Iodate paper is used to indicate the removal of most of the SO<sub>2</sub>, but the last traces are considered absent only when the odor of SO<sub>2</sub> can no longer be detected. However, it is not absolutely necessary to remove completely the SO<sub>2</sub>, though the presence of appreciable amounts will cause inconvenience. With the complete removal of SO<sub>2</sub>, iodide remains as the only substance in appreciable amounts that can reduce iodate.

The solutions were then cooled and transferred to 500 cc. separatory funnels. At this point the solution is acid; the chromium is present in the chromic state and the iodine as iodide. The amount of iodine usually present at this stage is little more than that added as carrier; namely, 2.5 mg. 10 to 15 cc. of CCl<sub>4</sub> along with 1 cc. of 0.02 M KIO<sub>3</sub> were added and the I<sub>2</sub> formed shaken out. Two more

<sup>4</sup> 1 cc. of 0.02 M KI yields about 5 mg. of AgI, a convenient amount for later mounting of the sample for measurement of its radioactivity.

extractions with  $\text{CCl}_4$  were carried out to complete the removal of iodine. The  $\text{KIO}_3$  used is 5 times the amount needed to oxidize the iodide to iodine. As a result, approximately four-fifths of the iodate remains behind. That there is no appreciable interchange between the radioactive  $\text{I}_2$  and the non-radioactive iodate under these conditions was shown as follows: After the labeled iodine was removed in the manner described, enough non-radioactive iodide was added to convert the excess iodate into iodine. This was then extracted and its radioactivity compared with that of the first iodine removed. The radioactive iodine thus found (less than 0.2 per cent) showed that the loss due to interchange or other factors is entirely negligible.

The iodine in  $\text{CCl}_4$  solution was titrated with  $\text{Na}_2\text{S}_2\text{O}_3$ . The iodide in the water phase was removed by several water washings and these made up to a volume of 50 or 100 cc., depending upon the estimated radioactivity present. Suitable aliquots were pipetted into 50 cc. beakers, more KI carrier added if necessary,<sup>4</sup> and the solution treated with an excess of  $\text{AgNO}_3$ . After the solution was heated on the steam bath until well coagulated, the  $\text{AgI}$  was filtered out upon 5.5 cm. No. 50 Whatman paper placed in a long stemmed funnel. The precipitate was dried with acetone and ether, and, if reasonable care is taken,  $\text{AgI}$  is present only on a semicircle of the filter paper. The precipitate was mechanically distributed over an area of 5 to 6 sq. cm. by means of a large steel needle, after which a piece of Scotch tape was placed over the sample. After the excess paper and tape were trimmed off, the resulting sample was a rectangle measuring  $\sim 3 \times 5$  cm. The samples were wrapped around a thin wall Geiger counter and the radioactivity determined in a manner previously described (2).

The labeled iodine extracted from the tissues is expressed as a percentage of the administered radioiodine. For this purpose a standard was prepared, which consisted of a portion of the same solution of labeled iodine as was administered to the animal. Its iodine was precipitated and mounted in a manner identical with that employed for tissues.

Because of the avidity of thyroid tissue for iodine, the radioactivity in a *few mg.* of it may be enough for an accurate measurement. This is especially true of rat thyroid, since the entire organ in this animal may weigh 15 mg. or less. In such cases the

lengthy process of iodine isolation may be avoided; the gland can be mashed on a non-absorbing surface and its radioactivity directly measured. The self-absorption of the radioactivity by the sample is small and can be made uniform. This procedure has been used to determine the uptake of radioiodine by rat thyroid shown in Fig. 4. The fresh glands were weighed immediately after excision and placed upon a piece of aluminum-foil measuring  $3 \times 5$  cm. that had been covered with a strip of lens paper on one side and blotting paper on the other. The lens paper prevents the thyroids from sliding while being mashed, while the blotter acts merely as a support for mounting. It is then wrapped in thin cellophane which is fixed with glue. By means of a smooth glass bottle the glands are rolled into a paste covering about 5 to 6 sq. cm. Necessary precautions were taken in the preparation of standards to rule out possible self-absorption resulting from the 10 to 15 mg. of thyroid tissue on the aluminum-foil.

*Distribution of 0.5 Mg. of Administered Labeled Iodine in Tissues of the Rat*

Each of twenty male rats weighing between 180 and 200 gm. received by stomach tube 1 cc. of an aqueous solution of labeled KI containing 0.5 mg. of iodine. The animals were kept in specially constructed monel cages mounted on large monel funnels equipped with a device for the separation of urine and feces (9). Access to food was permitted throughout the period of observation. At intervals of 1, 3, 6, 12, and 24 hours after the administration of the labeled iodine, the animals were anesthetized with nembutal and then bled. The thyroid glands were then quickly removed. The abdominal cavity was opened, the bladder clamped at its lower end, and its contents added to the urine sample obtained while the animal was kept in the cage. The small intestine and the stomach were washed with several 5 cc. portions of isotonic saline and the combined washings analyzed for unabsorbed iodine. The cecum and large intestine were added to the feces. The liver, kidney, and small intestine were then removed. The entire procedure required 6 to 8 minutes for its completion.

As noted above, the amount of labeled iodine received by each rat was 0.5 mg. *This far exceeds the normal iodine content of the entire animal.* It is estimated that between 0.1 and 0.2 mg. of

iodine are present in the whole rat (10). Since the dilution of the administered labeled iodine by the non-radioactive iodine already in the animal is very slight, it would appear that the specific activity of the total iodine entering the tissue is not much below that of the iodine administered.

*Absorption of Iodine from Gastrointestinal Tract*—The absorption of a relatively large dose of iodine from the gastrointestinal

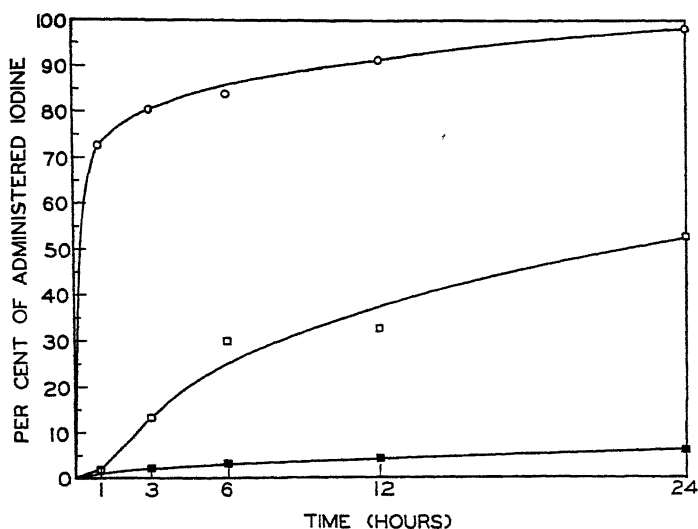


FIG. 1. The absorption and excretion of 0.5 mg. of labeled iodine as KI in the rat. All values are expressed as the per cent of the administered labeled iodine.  $\circ$  shows absorption, *i.e.* the difference between the amount administered and the amount found in the stomach and small intestine;  $\square$  urine;  $\blacksquare$  feces. Each point represents the average of four separate analyses on as many animals.

tract is shown in Fig. 1. Over 70 per cent of the administered iodine was absorbed at the end of the 1st hour. In 3 hours 80 per cent of the administered iodine had left the gastrointestinal tract, whereas in 12 hours absorption was 90 per cent complete. 1 per cent of the iodine was found in the small intestine at the end of 24 hours.

*Excretion in Urine and Feces*—When large doses are administered, iodine appears rapidly in the urine; an average of 13 per cent



of it was eliminated by this route 3 hours after its introduction into the stomach. At the 12 hour interval 33 per cent was found in the urine and 50 per cent at the end of 24 hours.

The samples called "feces" included the cecum and lower parts of the intestinal tract along with their contents. The values shown in Fig.1 are maximal, for the possibility that feces are contaminated with urine cannot be ruled out. At the 24 hour interval two animals had eliminated 10 per cent of the iodine by this route, whereas in two others values below 5 per cent were observed.

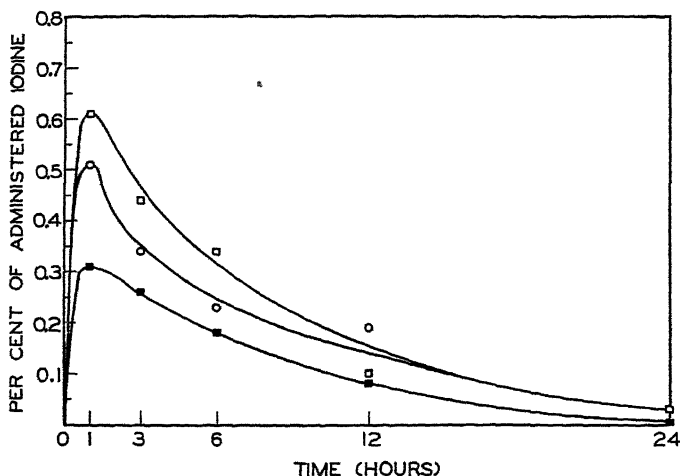


FIG. 2. The uptake of labeled iodine per gm. of small intestine, kidney, and liver of the rat. □ small intestine; ○ kidney; ■ liver. Each animal received 0.5 mg. of labeled iodine as KI. Each point represents the average of four separate analyses on as many animals.

At all other examinations, namely 1, 3, 6, and 12 hours after the administration of the labeled iodine, 5 per cent or less was found in the "feces."

*Uptake of Iodine by Liver*—This tissue is characterized by a rapid uptake and loss of iodine (Fig. 2). The maximum amount of iodine was found 1 hour after its administration, when about 0.3 per cent of the administered labeled iodine was present in each gm. of hepatic tissue. At the 12 hour interval less than 0.1 per cent per gm. was present in this tissue, and at the end of 24 hours it had almost disappeared.

*Uptake of Iodine by Small Intestine*—The course of iodine deposition and release in this part of the gastrointestinal tract resembles that in the liver (Fig. 2). The maximum deposited, however, was somewhat higher; namely, 0.6 per cent of the administered labeled iodine.

*Uptake of Iodine by Kidney*—The rise and fall of the iodine content of the kidney is very much like that found in the liver and small intestine (Fig. 2). Thus the maximum occurred at 1 hour, at which time about 0.5 per cent per gm. had been deposited. At

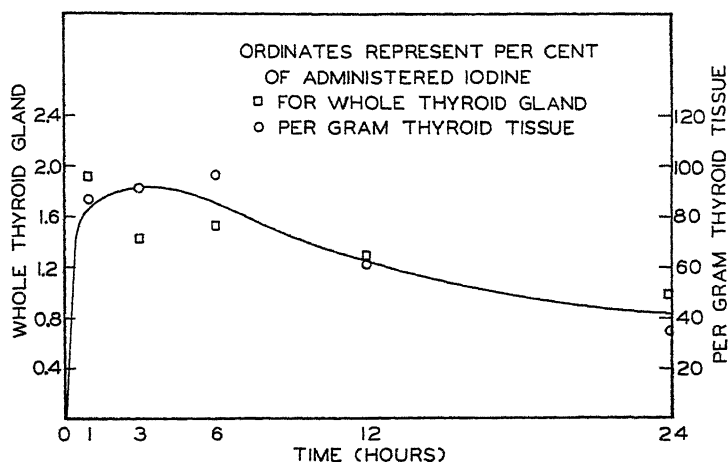


FIG. 3. The uptake of labeled iodine by the thyroid gland of the rat. Each animal received 0.5 mg. of labeled iodine as KI. Each point represents the average of four separate analyses on as many animals.

the 24 hour interval the content of labeled iodine was reduced to values below 0.05 per cent per gm.

*Uptake of Iodine by Thyroid Gland*—In none of the above tissues (liver, kidney, and small intestine) did the uptake of iodine exceed 1 per cent *per gm. of tissue*. When comparisons are made on this basis, thyroid tissue retains over 100 times as much as those tissues (Fig. 3). This was found to be true despite the fact that each rat received 0.5 mg. of labeled iodine, an amount far larger than that normally contained in the whole animal. Thus thyroid gland deposited about 100 per cent of the administered labeled iodine *per gm. of tissue* during the 3 to 6 hour interval. Similar

differences between the activities of thyroid and other tissues have been observed by Hertz, Roberts, and Evans (4).

The loss of deposited labeled iodine occurs slowly in the thyroid gland. At the 24 hour interval the gland still retained about one-half of the maximum amount found at the earlier periods. This slow rate of loss in the thyroid is in marked contrast to the loss of labeled iodine observed in the liver, kidney, and small intestine, in which practically none of the labeled iodine remained at the end of 24 hours.

*Turnover of Endogenous Iodine As Shown by Administration of Tracer Doses of Labeled Iodine*

*Thyroid Gland of Rat*—Young rats weighing approximately 120 gm. were fed labeled iodine by stomach tube, and thereafter groups of four were sacrificed at the following intervals, 0.25, 0.5, 1, 2, 5, 12, 24, 48, 96, 192, and 384 hours. The glands were mounted directly on the aluminum-foil, as described above.

The turnover of different amounts of labeled iodine by the whole thyroid is compared in Fig. 4. Each of the rats in the groups shown in the lower curve received by stomach tube 1 cc. of a solution of KI containing 0.03 mg. of I. This amounted to 0.25 mg. of iodine per kilo. This radioactive iodine was prepared by adding a carrier. Each animal of the group recorded in the upper curve received 1 cc. of a solution containing tracer amounts of radioiodine. As noted above, this solution did not contain measurable amounts of iodine; hence its administration did not alter the iodine content of the body. The tracer amounts of the radioiodine serve to label the iodine already present within the animal; hence the turnover of the radioiodine is indicative of the turnover of endogenous iodine.

Fig. 4 shows very strikingly that the uptake and loss of radioiodine by the thyroid gland differed for the two doses employed. In the case of the tracer dose, which, as already noted, contained negligible amounts of iodine, a maximum of 65 per cent of the administered radioiodine found its way into the *whole thyroid gland* between the 25 and 50 hour intervals. The retention of such a large part of the administered iodine by an amount of tissue that at no time weighed more than 15 mg. (average 10 mg.) is indeed surprising in view of the fact that the normal gland con-

tains only 20 per cent of the total body iodine (1, 10). The physiological interpretation of this finding will be dealt with below.

It is also of considerable interest to note that the curve depicting the turnover of the tracer dose of iodine is characterized by a sharp rise. As early as 1 hour after the administration of the radioiodine, 12 per cent of it was found in thyroid tissue. So rapid is the deposition in this tissue that over 50 per cent was deposited in 5 hours. Curiously enough, the release of radioiodine from the gland occurred much more slowly than the initial

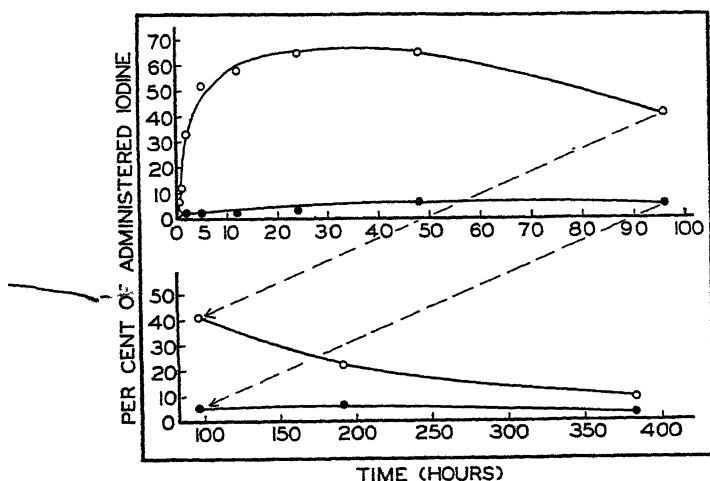


FIG. 4. The uptake of labeled iodine by the thyroid gland of the rat. O, each animal received a tracer dose of labeled iodine; ●, each animal received 0.03 mg. of labeled iodine. Each point represents the average of four separate analyses on as many animals.

uptake. Although a maximum deposition of 65 per cent was found as early as 24 hours, over 40 per cent was still in the gland as late as 96 hours and 20 per cent as late as 192 hours.

The lower curve of Fig. 4 shows that the inclusion of as little as 0.03 mg. of iodine as carrier greatly depressed the percentage of the administered radioactive iodine retained by the gland. The rats that received this dose of labeled iodine showed a maximum retention of 7 per cent in their whole thyroid glands. The curve depicting the turnover of this dose of iodine differed fundamentally from that noted in the case of the tracer dose. There was a slow

but steady accumulation of labeled iodine. Although the maximum of 7 per cent was observed at 50 hours, practically the same amount was still present at the 100 hour interval. Moreover, little change in the amount deposited was found at 192 hours; at this time 6 per cent of the administered labeled iodine was still present in the gland.

*Distribution of Tracer Dose of I\* in Tissues Other Than the Thyroid*—The percentage of the administered labeled iodine found in kidney, blood, skin, testes, liver, adrenal, muscle, and brain of the rabbit at intervals of 5 to 197 hours after the subcutaneous

TABLE I

*Distribution of Tracer Dose of I\* in Tissues of Rabbit Other Than Thyroid*

All values are expressed as percentages of the administered labeled iodine  $\times 10^2$ .

Each animal received subcutaneously an isotonic solution of NaCl containing the tracer amounts of I\*.

Tissues	Hrs. after I* injection				
	5	25	48	96	197
Kidney.....	8.10	0.592	0.539	0.400	0.207
Blood.....	5.57	0.544	0.421	0.262	0.248
Skin.....	3.68	0.281	0.222	0.179	0.131
Testes.....	2.47	0.605	0.576		0.228
Liver.....	2.43	0.289	0.158	0.117	0.0037
Adrenals.....	1.84	0.390	0.273	0.070	0.114
Muscle (gastrocnemius).. <td>1.05</td> <td>0.073</td> <td>0.056</td> <td>0.050</td> <td>0.038</td>	1.05	0.073	0.056	0.050	0.038
Brain.....		0.069	0.029	0.016	0.0097

injection of tracer doses of I\* is shown in Table I. The rabbits used in this study weighed 2 kilos. The labeled iodine was administered in an isotonic solution of sodium chloride; each animal received 3 cc. of this solution. At each time interval, the tissues of three rabbits were pooled, thoroughly mixed, and, whenever possible, 10 gm. samples were taken for analysis of their I\* content. In the case of blood, however, 2 cc. were removed from each animal.

It should again be noted that this distribution reflects the movement of endogenous iodine, since the injected iodine could not have increased the amount already present within the animal. The tissue that showed the highest concentration of the labeled iodine

at the early interval (5 hours) was kidney. At this time  $8.1 \times 10^{-2}$  per cent of the injected  $I^*$  was present in each gm. of kidney; but so temporary is its storage in this tissue that by 25 hours the amount present was reduced to  $0.59 \times 10^{-2}$  per cent. Blood contained a little less than kidney at the 5 hour interval, but thereafter the concentrations found in blood were very close to those in the kidney. Skin, testes, adrenal, and liver contained between  $2 \times 10^{-2}$  and  $4 \times 10^{-2}$  per cent of the injected  $I^*$  per gm. of tissue at the 5 hour period. The movement of circulating iodine occurs at lower levels *in* and *out* of muscle and brain than in and out of the other tissues listed above. Although the content of  $I^*$  in muscle was only slightly below that of adrenal at the 5 hour interval, its concentration dropped considerably below that of other tissues at the 48 and 96 hour intervals.

#### DISCUSSION AND SUMMARY

*Thyroid*—In the present investigation the uptake of radioiodine by the rat's thyroid gland was examined after the administration of three separate amounts of labeled iodine: 0.5 mg., 0.03 mg., and an amount that has been termed here a tracer dose, *i.e.* one in which the radioiodine contains no carrier and hence is mixed with a negligible amount of iodine. For the purposes of the following comparison it will be assumed that the thyroid glands of the rats were of uniform size.<sup>5</sup> When each rat received 0.5 mg., the maximum concentration of the administered labeled iodine found in the *whole* gland was about 2 per cent. When each animal received 0.03 mg., about 7 per cent of the administered labeled iodine was the maximum that entered the gland. But when a tracer dose was injected, 65 per cent of the radioiodine appeared in the gland.

Since the introduction of the tracer dose does not alter the total iodine content of the body, it serves as a labeling device for circulating iodine as soon as it is completely mixed with blood iodine. It would therefore appear that the distribution of the tracer dose reflects the movement of circulating endogenous iodine. The appearance of 65 per cent of the tracer dose in as small an amount of tissue as 15 mg. leaves no doubt of the capacity of the thyroid gland to sweep iodine out of the circulation. Furthermore, since

<sup>5</sup> Their weights did vary from 10 to 15 mg., but this variation has no bearing on the points made.

it has been shown that the thyroid gland holds only 20 per cent of the total body iodine (1, 10), the uptake of as much as 65 per cent of the tracer dose by this gland suggests that circulating iodine is removed by it much faster than it can come into equilibrium with tissues other than the thyroid.

It should not be inferred from these findings that a larger *amount* of iodine is removed from the circulation by the gland when a tracer dose is employed than when 0.5 or 0.03 mg. is injected. No dilution of blood iodine occurs when the tracer dose is used. When the other amounts are administered, particularly 0.5 mg., the amount of circulating iodine is undoubtedly increased. In each case a fraction of the circulating iodine enters the gland, but in each case a unit of radioactivity represents a different quantity of blood iodine. Since these amounts of circulating iodine are not known, the percentages of the administered labeled iodine found in the glands cannot be expressed in mg. of iodine. Although a dilution mechanism is suggested as an explanation for the differences observed in the uptake of the thyroid gland when the above three doses of labeled iodine were employed, it should nevertheless be noted that no proof for this view is offered. This must await careful and detailed blood iodine measurements.

In view of the rapid entrance of labeled iodine into the gland, it is somewhat surprising to find that its release from this tissue takes place at a remarkably slow rate. This fact is particularly well brought out in the experiments in which rats received 0.03 and tracer amounts of labeled iodine (Fig. 4), for in these cases observations on the iodine content of the glands were extended to 360 hours. When the former dose was employed, maximum concentration of radioiodine appeared as early as 48 hours, but this same amount was still present in the gland as late as 192 hours. Even at 384 hours more than half the amount found in the gland at 48 hours was still present. The story for the tracer dose is not much different. In this case the maximum concentration of radioiodine appeared as early as 24 hours; namely, 65 per cent of the administered labeled iodine. But at 96 hours 40 per cent was still contained in the thyroid gland, and at 192 hours over 20 per cent remained in the whole gland. The simplest, though not necessarily the only, explanation of this phenomenon (*viz.* the rapid uptake as compared with a very slow release) would appear to

lie in the fact that the labeled iodine entering the gland is greatly *diluted* by the relatively large amounts of iodine already present in the normal gland. If, on the other hand, the entering radioiodine had comprised a large part of the total thyroid iodine, its rapid entrance would have been followed by an equally rapid release.

*Tissues Other Than Thyroid*—Although the amounts of labeled iodine that appeared in liver, small intestine, kidneys, muscle, adrenals, testes, skin, blood, and brain differed widely at the early intervals, its rate of removal from these tissues was roughly similar. Maximum concentrations were always observed in these tissues at the first interval, when the concentration of labeled iodine in the blood was highest. The parallelism observed in the loss of labeled iodine by these tissues and by blood suggests a diffusion of iodine in and out of these tissues rather than a selective retention.

The samples of radioactive iodine used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

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# RADIOACTIVE IODINE AS AN INDICATOR OF THE METABOLISM OF IODINE

## II. THE RATES OF FORMATION OF THYROXINE AND DIIODOTYROSINE BY THE INTACT NORMAL THYROID GLAND\*

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According to Harington, two fractions, one "thyroxine-like" and the other "diiodotyrosine-like," can account for all of the organic iodine found in the thyroid gland (1). The metabolism of these compounds has been reviewed recently by Salter (2). The labeling of iodine by radioactivity provides for the first time a desirable tool for the study of the endogenous or normal rates of formation of these fractions. In the present investigation tracer doses of radioiodine (3) were administered to normal sheep and rats, and at various intervals thereafter the amounts of labeled thyroxine and diiodotyrosine deposited in the thyroid gland were measured.

### EXPERIMENTAL

*Sheep*<sup>1</sup>—The sheep employed weighed approximately 35 kilos. Each animal was injected intraperitoneally with 3 cc. of an isotonic solution of saline containing tracer amounts of radioiodine. The labeled iodine was prepared in the manner previously described (3). The thyroid glands were removed at two intervals, 4 and 48 hours after the iodine injections; they were immediately

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<sup>1</sup> Our thanks are due to Dr. R. J. Tompkins and to the Golden West Meat Company, Emeryville, for the supply of sheep glands.

frozen in CO<sub>2</sub> snow and kept so until analyzed. Before removal of the glands, the animals were bled.

*Rats*—Radioactive iodine, in tracer doses, was fed by stomach tube to male rats weighing 160 to 180 gm. At intervals of 2, 4, 48, and 96 hours, groups of four rats were sacrificed and their thyroids quickly removed, weighed, and placed in flasks for hydrolysis. To each flask were added 250 mg. of desiccated thyroid and 25 cc. of 2 N NaOH, and the mixture was heated on a steam bath for 8 hours. The sheep thyroids were treated in a similar manner except that the addition of desiccated thyroid as carrier was not necessary.

The hydrolysates were made up to a volume of 50 or 100 cc. and suitable aliquots removed for fractionation as well as for the determination of the total iodine radioactivity. For the latter purpose, the solution was ashed and its iodine isolated in the manner previously described (3).

*Fractionation of Thyroid Iodine*—The thyroid hydrolysate was separated into three iodine-containing fractions by a modification of the procedures devised by Leland and Foster (4) and by Blau (5). Butyl alcohol was used for the separation of thyroxine and diiodotyrosine. Inorganic iodine was removed from the fraction insoluble in butyl alcohol. Throughout the procedure "carriers" were used to advantage, since only the radioactivity of the various fractions was measured.

An aliquot of the alkaline thyroid hydrolysate is adjusted to pH 3.5 to 4, according to Blau's method (5), and vigorously shaken in a separatory funnel with an equal volume of butyl alcohol. Complete separation of water and alcohol phases occurs in about 6 hours (they may be left overnight at this stage). The aqueous layer is drained off into a second separatory funnel, and to this is added about 1 mg. of inert thyroxine<sup>2</sup> as carrier. A second butyl alcohol extraction is then made with 0.5 volume of butyl alcohol. Both alcoholic extracts are now combined in a separatory funnel and to it are added approximately 1 mg. of diiodotyrosine, 1 cc. of 0.02 M KI, and an amount of 20 per cent technical NaOH equal in volume to the combined alcoholic extract. This extraction in the

<sup>2</sup> The thyroxine used in this study was generously donated by E. R. Squibb and Sons through the courtesy of Dr. G. A. Harrop and Dr. H. Jensen.

presence of KI and diiodotyrosine as carriers served to remove by dilution any radioactive inorganic iodine and diiodotyrosine that may be occluded or dissolved in the butyl alcohol. This treatment of the butyl alcohol is repeated with 0.5 volume of 20 per cent NaOH. Both aqueous alkaline washings are now combined with the aqueous residue of the butyl alcohol extraction; this contains the inorganic iodine and the diiodotyrosine. Thyroxine is present in the butyl alcohol layer.

The butyl alcohol extracts are transferred to a Kjeldahl flask and concentrated to complete dryness under reduced pressure at 55°. The residue is ashed; its iodine is isolated and radioactivity determined in the manner previously described (3).

The combined aqueous extracts are made acid to methyl orange with  $\text{H}_2\text{SO}_4$ . 1 cc. of 0.02 M  $\text{KIO}_3$  is added and the resulting iodine quickly extracted with carbon tetrachloride. Three extractions are sufficient for its complete removal. The carbon tetrachloride washings now contain all the inorganic iodide of the thyroid hydrolysate. The remaining aqueous layer, which still holds the diiodotyrosine fraction, is ashed without further treatment; 20 cc. of 50 per cent  $\text{CrO}_3$  solution and 40 cc. of 1:1  $\text{H}_2\text{SO}_4$  were added for each 20 cc. of original hydrolysate.

To determine the reliability of the method, the following tests were made. (1) Small amounts of radioactive iodide were added to samples of desiccated thyroid. The mixture was hydrolyzed with NaOH and fractionated in the manner described above. The radioactivity was quantitatively recovered in the carbon tetrachloride layer. This shows that no loss of inorganic iodine occurred during hydrolysis and separation, and that no iodide was organically bound during the process. (2) Radioactive diiodotyrosine was synthesized according to the procedure of Harington (6) by the use of radiiodide. A few mg. of this preparation were added to desiccated thyroid and the mixture carried through the above procedure. No radioactivity was found in the butyl alcohol fraction; 3 per cent of it, however, appeared in the inorganic fraction. Apparently no synthesis of thyroxine occurs during the procedure, but a small fraction of the diiodotyrosine does break down to inorganic iodide.

Although it was not found possible to prepare radioactive thyroxine of sufficient strength to test its recovery, it is neverthe-

less clear that the thyroxine values obtained by this method of fractionation are low, since it is not synthesized from iodide or diiodotyrosine and some of it is probably destroyed (Blau (5)). The diiodotyrosine values obtained by this fractionation are not necessarily low, since its breakdown is slight and some iodine may appear here from the destruction of thyroxine. The values obtained by the present procedure for inorganic iodine are probably a little high because of the slight breakdown of diiodotyrosine noted above.

### Results

*Sheep Thyroid*—Measurements of the various labeled fractions in the gland were made at two intervals after the feeding of tracer amounts of labeled iodine; *i.e.*, at 4 and 48 hours. The values are recorded in Table I. Considerable differences were observed in the gland's uptake of labeled iodine at each interval. Thus between 2 and 12 per cent of the administered labeled iodine was found in both glands of the sheep at the earlier interval; the values at 48 hours were as high as 39 per cent. Both glands in these animals weighed 2 to 2.8 gm.; the total body weight was 35 kilos. As much as 10 per cent of the administered labeled iodine was found as diiodotyrosine at the 4 hour period; as much as 30 per cent was in this form 48 hours after the  $I^*$  administration. Much less of the labeled element found its way into the "thyroxine-like" fraction: less than 1 per cent at 4 hours and a maximum of 3.4 per cent at 48 hours.

Despite these fluctuations in the actual amounts of labeled iodine uptake by the gland, its partition, *as measured by the percentage of the total thyroid  $I^*$  deposited as labeled thyroxine and diiodotyrosine*,<sup>3</sup> remained fairly constant at each interval. Thus

<sup>3</sup> Fluctuations in the actual amounts or percentages of the administered labeled iodine that found their way into the two fractions, thyroxine and diiodotyrosine, could be due in part to differences in the amounts of circulating inorganic iodide present in these animals at the time of injection of the radioiodine. As already noted in Paper I (3), the tracer doses of radioiodine serve to label the circulating iodine as soon as it mixes with blood iodine. It is the amount of circulating iodine that determines the percentage of circulating iodine removed by the gland for conversion to thyroxine and diiodotyrosine. For this reason the relative distribution of total labeled thyroid iodine between the two organic fractions is a more significant measurement than the actual percentage of the administered radioiodine found either as thyroxine or as diiodotyrosine.

4 hours after the administration of I\*, 82 to 88 per cent of the total labeled thyroid iodine was present in the glands as diiodotyrosine; from 5 to 7 per cent as thyroxine. Despite the increased uptake of total I\* at 48 hours as compared with the 4 hour interval, the proportion of total thyroid I\* incorporated in the thyroxine and diiodotyrosine changed but little; at 48 hours 76 to 80 per cent of thyroid iodine was present in the diiodotyrosine fraction, 8 to 9 per cent as thyroxine.

*Rat Thyroid*—The weights of the rat thyroids varied from 10 to 21 mg. The values shown in Table II for labeled iodine or frac-

TABLE I  
*Sheep Thyroids*

Animal No.	Time after I* administration	Weight of glands	Per cent of administered I* recovered in whole thyroid gland					Per cent of total thyroid I* found as		
			Total determined (1)	As thyroxine (2)	As diiodotyrosine (3)	As inorganic (4)	Total recovered (2) + (3) + (4)	Thyroxine	Diiodotyrosine	Inorganic
	<i>hrs.</i>	<i>gm.</i>								
7	4	2.08	2.28	0.12	1.99	0.20	2.31	5.4	86.0	8.7
8	4	2.06	13.00	0.95	10.58	1.32	12.85	7.5	82.3	10.3
9	4	2.82	11.90	0.71	10.00	0.63	11.33	6.3	88.2	5.5
10	48	1.95	25.50	2.26	20.70	3.24	26.20	8.6	79.0	12.4
11	48	2.66	38.80	3.44	29.80	4.29	37.53	9.2	79.5	11.4
12	48	1.52*	10.48*	0.79*	7.40*	1.52*	9.71*	8.1	76.3	15.7

\* These values do not represent the content of the whole gland. It was severed during its removal from the neck.

tions thereof represent the amounts found in the whole of the thyroid gland removed from each rat. 11 to 17 per cent of the fed labeled iodine was found in the thyroid glands of the rat at the 2 hour interval. Larger amounts were deposited at 48 and 96 hours. Thyroxine is rapidly formed in rat thyroid. 1.5 to 3 per cent of the administered I\* was found as thyroxine as early as 2 hours and from 1.5 to 5 per cent at the end of 4 hours. The amounts of thyroxine found at 48 and 96 hours varied considerably; the highest value observed was 16 per cent.

In all cases a larger percentage of the I\* was found as diiodotyrosine than as thyroxine. Uniform amounts as diiodotyrosine were not found at either 4 or 48 hours. At 4 hours the lowest and

highest values were respectively 7 and 17 per cent. At 48 hours as little as 7 per cent and as high as 32 per cent of the administered labeled iodine appeared as diiodotyrosine.

It is again of interest to note that despite the marked fluctuations in the amounts of administered labeled iodine deposited as either thyroxine or diiodotyrosine, the proportion of the total radioactive thyroid iodine deposited in each fraction remained

TABLE II  
*Rat Thyroids*

Rat No.	Time after I* administration	Weight of glands	Per cent of administered I* recovered in whole thyroid gland					Per cent of total thyroid I* found as		
			Total determined (1)	As thyroxine (2)	As diiodotyrosine (3)	As inorganic (4)	Total recovered (2) + (3) + (4)	Thyroxine	Diiodotyrosine	Inorganic
	hrs.	mg.								
22	2	14.8	10.8	1.56				14.4		
23	2	17.1	17.3	3.18				18.4		
24	2	16.1	12.9	1.73				13.4		
25	2	20.9	15.3	1.94				12.7		
14	4	19.3	25.7	4.73	17.40	2.08	24.2	18.4	67.7	8.1
15	4	15.8	16.2	3.52	11.30	1.41	16.2	21.7	69.8	8.7
16	4	18.5	13.4	2.47	9.80	1.21	13.5	18.4	73.1	9.0
17	4	16.5	10.5	1.68	7.39	0.90	9.9	16.0	70.4	8.6
18	48	11.1	32.4	9.82	17.90	3.18	30.9	30.6	55.2	9.8
19	48	15.2	28.6	9.63	15.60	3.74	29.0	33.7	54.4	13.1
20	48	18.8	10.7	3.25	6.70	1.02	10.9	30.4	62.7	9.5
21	48	19.1	52.2	15.80	31.80	4.17	51.8	30.3	60.9	8.0
26	96	13.2	24.6	5.61				22.8		
27	96	10.5	37.3	8.85				23.7		
28	96	13.5	14.0	3.64				26.0		
29	96	13.6	15.7	4.04				25.7		

rather constant at each time interval. Thus, despite fluctuations in actual percentage of I\* from 7.4 to 17.4 in the case of diiodotyrosine and from 1.7 to 4.7 in the case of thyroxine at the 4 hour interval, these values represented from 68 to 70 per cent of the total labeled thyroid iodine in the case of diiodotyrosine and from 16 to 22 per cent of the total thyroid I\* in the case of thyroxine. This proportional distribution of the labeled thyroid iodine was not the same, however, at all intervals. The results indicate that a larger

fraction of the total labeled thyroid iodine is present in the form of thyroxine at 48 hours than at 4 hours. It is of interest to note here that, in reviewing the earlier work on radioiodine, Salter states that the thyroxine-like and diiodotyrosine-like fractions are deposited at a ratio of 1:2 in thyroids of patients suffering from Graves' disease (2).

#### SUMMARY

The rates at which thyroxine and diiodotyrosine are deposited in the normal thyroid glands of sheep and rats were measured with radioactive iodine as indicator. Tracer amounts of labeled iodine (*i.e.* samples containing practically no iodine) were employed. Hence, the rates recorded are indicative of *endogenous formation*.

1. Thyroxine is rapidly formed in the thyroid gland of both rats and sheep. As early as 2 hours after I\* administration to rats weighing 160 to 180 gm., 1.5 to 3 per cent of it was retained as thyroxine in the entire thyroid glands that weighed only between 11 and 21 mg. 4 hours after its intraperitoneal injection in sheep weighing 35 kilos, 0.1 to 0.9 per cent of the labeled iodine was found deposited in the whole thyroid glands (2 gm.). Increased amounts of the administered I\* appeared in this fraction at later intervals, and in a single case as much as 16 per cent was deposited as thyroxine in 19 mg. of rat thyroid in 48 hours.

2. A larger percentage of the administered I\* was found as diiodotyrosine than as thyroxine at all intervals in both rats and sheep. From 7 to 17 per cent of the administered labeled iodine was deposited as diiodotyrosine in rat thyroids in 48 hours; as high as 32 per cent of the labeled iodine was found in this fraction at the end of 48 hours.

3. Despite a good deal of fluctuation in the actual amounts of labeled iodine deposited as thyroxine and diiodotyrosine in the gland, *the proportion of the total labeled thyroid iodine represented by each of these fractions remained fairly constant at each time interval.*

The samples of radioiodine used in this investigation were prepared in the Berkeley cyclotron by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due. We are also grateful to Dr. J. G. Hamilton for the isolation of the iodine without carrier.



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## CONCERNING THE CHEMICAL COMPOSITION OF CYSTICERCUS FASCIOLARIS

### II. THE OCCURRENCE OF A CEREBROSIDE CONTAINING DI- HYDROSPHINGOSINE AND OF HYDROLECITHIN IN CYSTICERCUS LARVAE\*

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In a recent investigation on the chemical composition of *Cysticercus fasciolaris* Salisbury and Anderson (1) found that about 34 per cent of the lipids extracted with alcohol-ether and chloroform, following extraction with acetone, were insoluble in anhydrous ether. Preliminary examination of the ether-insoluble substance indicated that it was a mixture of cerebroside and phospholipid.

We have studied more closely the ether-insoluble portion of the lipids of *Cysticercus fasciolaris* and have succeeded in separating this material into a cerebroside which was practically free from phosphorus and into a phospholipid which contained only a trace of cerebroside. The purification and chemical composition of these components form the subjects of the present report.

The cerebroside was found to be predominantly hydrophrenosin which on hydrolysis yielded galactose, phrenosinic acid, a small amount of lower fatty acids which could not be identified, and a saturated nitrogenous base that corresponded in composition to dihydrosphingosine. The presence of dihydrosphingosine in a naturally occurring cerebroside is quite unusual, because all previously described cerebroside contain the unsaturated base sphingosine.

The phospholipid was found to correspond in composition to a monoaminomonophosphatide but it contained a trace of cere-

\* This investigation has been supported by a grant from the International Cancer Research Foundation.

broside as impurity. On hydrolysis the substance gave fatty acids, glycerophosphoric acid, and choline; *i.e.*, cleavage products typical of lecithin. The fatty acids, however, differed from those of ordinary lecithin in that they were nearly completely saturated and consisted mainly of palmitic acid. In fact the substance was essentially a dipalmitolecithin. The occurrence of hydrolecithin in animal tissues is very unusual and so far as we know has not previously been reported.

The presence of hydrophrenosin and hydrolecithin in relatively large amounts in *Cysticercus* larvae would suggest that certain peculiar metabolic processes occur in this parasite. It would be of interest to determine whether the mature form of the parasite, *Taenia taeniaformis*, the common tapeworm of the cat, also contains the same constituents.

#### EXPERIMENTAL

The materials used in this investigation were generously provided by Dr. W. F. Dunning and Dr. M. R. Curtis of the Crocker Institute of Cancer Research, Columbia University. This material consisted of three different fractions, (a) the larvae of *Cysticercus fasciolaris* such as were used in the first investigation (1); (b) larvae plus the cyst membranes; (c) the cyst membranes alone. The fresh tissues were dried immediately over calcium chloride in vacuum desiccators from which the air had been displaced with carbon dioxide.

The dried fractions as received in the laboratory were first exhaustively extracted with acetone and later with alcohol-ether and finally with chloroform, as described in the former paper (1). The amounts of lipids obtained are given in Table I and are calculated on a water-free basis.

*Isolation of Ether-Insoluble Lipids*—The lipids contained in the acetone extracts were not examined, since it was found and reported in a former paper (1) that this material consisted mainly of cholesterol. The lipids extracted with alcohol-ether and with chloroform were combined and dissolved in a small volume of chloroform. The solution was diluted with 15 volumes of anhydrous ether and cooled in the refrigerator overnight. A yellowish amorphous precipitate which separated was centrifuged off and washed with ether. The mother liquor on concentration to

a small volume, dilution with anhydrous ether, and cooling gave an additional small amount of precipitate which was collected, washed with ether, and combined with the first lot.

The mother liquors on concentration to dryness left residues which were easily and completely soluble in ether. The ether-soluble lipids from the three source materials were recovered separately and reserved for future investigations.

The quantities of ether-insoluble lipids obtained are shown in Table II.

TABLE I  
*Lipids and Ash in Cysticercus Larvae and in Cyst Membranes*

	Cysticercus larvae	Larvae plus cyst membranes	Cyst membranes
Dried material, gm.....	799	360	105.5
Ash, %.....		15.78	8.85
Acetone extract, gm.....	20.213	12.711	5.961
Alcohol-ether extract, gm.....	21.890	10.480	2.720
Chloroform extract, gm.....	0.702	0.634	0.034
Total lipids, gm.....	42.805	23.825	8.715
" " %.....	5.35	6.61	8.26

TABLE II  
*Ether-Insoluble Lipids*

	Cysticercus larvae	Larvae plus cyst membranes	Cyst membranes
Total lipids from alcohol-ether and $\text{CHCl}_3$ extracts, gm.....	22.592	11.114	2.754
Ether-insoluble lipids, gm.....	6.50	2.817	1.110
" " %.....	28.7	25.3	40.3

*Purification of the Ether-Insoluble Lipids*—For purification the ether-insoluble lipid fractions were dissolved in about 5 volumes of chloroform and warm alcohol was added to faint turbidity. Nearly white amorphous powders separated as the solutions were cooled to room temperature. The precipitates were filtered off, washed with cold alcohol, and dried *in vacuo*. The mother liquors on concentration and cooling gave additional small amounts of precipitates which were collected and combined with the first crop. The following amounts were obtained: (a) from *Cysticercus*

larvae 5.04 gm., m.p. 215–220° with decomposition, P 1.75 per cent; (b) from larvae plus cyst membranes, 1.81 gm., m.p. 215–220° with decomposition, P 1.76 per cent. These two fractions were combined and examined as will be described below. (c) The corresponding material from the cyst membranes weighed about 0.5 gm. It was of reddish color and contained 2.56 per cent of phosphorus; hence it was kept separate and has not yet been investigated.

*Separation of Cerebroside and Phospholipid*—The fractions (a) and (b) mentioned above appeared to be identical with the cerebroside-phospholipid mixture encountered by Salisbury and Anderson (1). The fractions were combined and precipitated first from chloroform solution by addition of ether and then from chloroform with addition of alcohol. These operations yielded a nearly white amorphous powder that weighed 5.57 gm.

Since we were under the impression that we were dealing with a mixture of cerebroside and sphingomyelin, we tried to separate the components by the procedure recommended by Rosenheim (2). The substance was dissolved in warm pyridine and the precipitate that separated on cooling to room temperature was filtered off and washed with pyridine. This fraction weighed 2.459 gm. and consisted of crude phospholipid. It was purified as will be described later. The filtrate and washings on evaporation to dryness *in vacuo* left a residue consisting of crude cerebroside that weighed 3.086 gm.

*Purification of Cerebroside*—The crude cerebroside was found to contain 0.4 per cent of phosphorus. In order to remove admixed phospholipid the substance was treated according to Klenk's (3) procedure in hot methyl alcoholic solution with cadmium acetate and the precipitate that separated was filtered off. The material recovered from the solution, after the excess of cadmium had been removed, was dissolved in a hot mixture of chloroform and methyl alcohol, 2:1. The cerebroside which separated on cooling was filtered off and washed with cold chloroform. For the final purification the substance was dissolved in hot methyl alcohol and as the solution cooled a white crust was deposited on the sides and bottom of the flask. The solid product, which showed no definite crystalline structure, was collected, washed with methyl alcohol, and dried *in vacuo*. It weighed 1.95 gm. and on

trituration formed a fine white powder. The substance was free from sulfur. Heated in a capillary tube the substance sintered at about 140°; and at about 170° somewhat opaque droplets, apparently liquid crystals, formed which melted to a clear fluid at 212–214°.

*Rotation*—0.1673 gm. of substance dissolved in 3 cc. of pyridine gave, in a 1 dm. tube,  $\alpha = +0.51^\circ$ ; hence  $[\alpha]_D^{25} = +9.1^\circ$

Mol. wt. (Rast) 810. Iodine No. (Hanus) 0.3

*Analysis*—Found, C 67.57, H 11.69, N 1.67, P 0.02

The values found agree approximately with the calculated composition of dihydrophrenosin plus 1 H<sub>2</sub>O. The small amount of phosphorus would indicate that the substance still contained about 0.5 per cent of phospholipid as an impurity.

The substance was evidently saturated, since practically no iodine was absorbed, and accordingly it represented an unusual type of cerebroside.

*Hydrolysis of Cerebroside*—The cerebroside, 1.8 gm., was rubbed into a fine suspension with 50 cc. of 5 per cent sulfuric acid and the mixture was refluxed for 26 hours. The insoluble material was filtered off, after the mixture had cooled, washed thoroughly with water, and dried *in vacuo*. The filtrate and washings were extracted with ether and the extract, after being washed with water, was dried over sodium sulfate, filtered, and evaporated to dryness. The residue was combined with the water-insoluble fraction and examined, as will be described later.

*The Water-Soluble Component. Identification of Galactose*—The aqueous solution gave a red color when heated with phloroglucinol and hydrochloric acid, but no coloration was obtained with orcinol, thus indicating the presence of galactose and absence of pentose. The sulfuric acid was removed quantitatively with barium hydroxide, after which the solution was concentrated *in vacuo* to a volume of about 4.0 cc. To the solution were added 1.5 cc. of absolute alcohol and 0.4 gm. of methylphenylhydrazine. The hydrazone began to crystallize immediately and after the solution had stood in the refrigerator overnight the crystals were filtered off, washed with cold 30 per cent alcohol, and dried *in vacuo*. The crude hydrazone, 0.49 gm., was recrystallized from 30 per cent alcohol after treatment with norit and gave 0.31 gm. of prac-



*Analysis*— $(C_{18}H_{35}O_2N)_2H_2SO_4(700)$

Calculated. C 61.71, H 11.14, N 4.00, OH 9.71,  $H_2SO_4$  14.00

Found. " 61.35, " 11.20, " 3.86, " 9.63, " 14.20

The amount of sulfuric acid was determined by titration in alcoholic solution with alcoholic potassium hydroxide, with phenolphthalein as indicator.

The analytical results indicate that the substance was dihydrosphingosine sulfate. The occurrence in a cerebroside of dihydrosphingosine is most unusual, since dihydrosphingosine has been obtained previously only by hydrogenation of sphingosine.

The constitution of sphingosine has apparently not been definitely established. The investigations of Klenk (5) and of Klenk and Diebold (6) would indicate that the old formula,  $C_{17}H_{35}O_2N$ , is incorrect but based on their work a new formula,  $C_{18}H_{37}O_2N$ , was proposed. If the new formula is correct, then dihydrosphingosine should be represented by the formula  $C_{18}H_{39}O_2N$ . It will be noted that the results obtained in our analysis agree more closely with the calculated values of the new than with the old formula.

*Examination of the Fatty Acids*—The fatty acids were isolated in the usual manner from the barium salts mentioned above and after being dried *in vacuo* weighed 0.73 gm., which is equivalent to 40.5 per cent of the cerebroside. The substance was dissolved in alcohol and separated according to the procedure of Klenk (7) by means of magnesium acetate. The acids recovered from the alcohol-insoluble magnesium salt weighed 0.518 gm. and those from the soluble magnesium salt weighed 0.20 gm.

*The Phrenosinic Acid*—The acid recovered from the insoluble magnesium salt was precipitated repeatedly from a mixture of hot alcohol-acetone, 1:1, by cooling slowly until the melting point of the acid was constant at 90–92°. The acid separated in the form of colorless, fine globular particles that showed no definite crystalline structure. The purified acid weighed 0.276 gm. and gave the following values on analysis.

*Rotation*—0.2110 gm. of acid dissolved in pyridine and diluted to 10 cc. gave, in a 1 dm. tube,  $\alpha = +0.09^\circ$ ; hence  $[\alpha]_D^{25} = +4.26^\circ$ .

*Analysis*— $C_{24}H_{48}O_3(382)$ . Calculated. C 75.00, H 12.50

$C_{25}H_{50}O_3(398)$ . " " 75.37, " 12.56

Found. " 75.16, " 12.39

Mol. wt. by titration 392



According to Chibnall *et al.* (8) phrenosinic acid as ordinarily obtained represents a mixture of several  $\alpha$ -hydroxy acids. The acid described above is evidently a mixture of hydroxy acids corresponding to what is usually called phrenosinic acid or cerebronic acid.

The fatty acid isolated from the alcohol-soluble magnesium salt was apparently a mixture from which no pure acid could be obtained. The acid did not crystallize, but separated from 70 per cent alcohol as a white amorphous powder. It melted at 57° and the molecular weight determined by titration was 297. On analysis the following values were found, C 76.71, H 12.81. It is evident from the analytical results that the substance was a mixture of lower acids and that very little lignoceric acid could have been present.

*Purification of the Phospholipid Fraction*—The crude phospholipid resembled sphingomyelin in solubility and it was therefore purified by the procedure recommended by Levene (9) for the preparation of pure sphingomyelin. The substance, 2.459 gm., was dissolved in 125 cc. of glacial acetic acid at 55° and the solution was allowed to stand at room temperature overnight. The slight precipitate which had separated was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in 100 cc. of a mixture of ligroin-absolute alcohol, 5:1, by warming, after which about 75 cc. of absolute alcohol were added and the cloudy solution was allowed to stand in the refrigerator overnight. A slight, dark colored precipitate which had separated was filtered off and the filtrate was evaporated *in vacuo* to dryness. The residue was dissolved in 80 cc. of 1:1 chloroform-pyridine at 50° and the solution was allowed to stand at room temperature overnight. The precipitate was filtered off, washed with ether, redissolved in 50 cc. of hot methyl alcohol and 50 cc. of pyridine were added. A nearly colorless precipitate, which separated as the solution was cooled, was filtered off and washed with pyridine and with ether. The substance was finally dissolved in 50 cc. of warm pyridine and the solution was cooled, whereupon a white amorphous precipitate separated which was filtered off, washed with pyridine and with ether, and dried *in vacuo* over sulfuric acid. The several mother liquors were concentrated and yielded additional small quantities of material

which was purified as mentioned above. The total yield of purified phospholipid amounted to 1.93 gm. and it was a white amorphous powder.

*Properties and Composition of Purified Phospholipid*—Heated in a capillary tube, the substance sintered at 72° and at 81° minute, semiliquid droplets formed, apparently liquid crystals, which melted with decomposition at 230–231°.

*Rotation*—0.1509 gm. of substance dissolved in chloroform and diluted to 3.0 cc. gave, in a 1 dm. tube,  $\alpha = +0.36^\circ$ ; hence  $[\alpha]_D^{20} = +7.1^\circ$

*Analysis*—Found, C 63.34, H 11.07, P 3.96, N 1.80. Iodine No. (Hanus) 4.4. Mol. wt. (Rast) 838

The nitrogen to phosphorus ratio, which was 1:1, and the low iodine number indicated that the substance was a monoamino-monophosphatide containing mainly saturated fatty acids.

*Hydrolysis of Phospholipid*—1.639 gm. of the substance were rubbed into a fine suspension in 60 cc. of 5 per cent sulfuric acid and refluxed for 6 hours. After the mixture had cooled, the fatty acids were filtered off, washed with water, resuspended in 50 cc. of 5 per cent sulfuric acid, and refluxed for 24 hours. That the hydrolysis was completed at this time was indicated by the fact that the fatty acids formed a clear oily layer on the surface of the hot aqueous solution. The fatty acids were filtered off after the solution had cooled and washed with water and examined, as will be described later.

*Examination of the Water-Soluble Constituents*—The two aqueous filtrates and washings mentioned above were combined and extracted with ether. The ethereal extract was washed with water, dried over sodium sulfate, filtered, and evaporated to dryness. The slight residue thus obtained was combined with the fatty acids.

The aqueous solution was concentrated *in vacuo*, made up to a volume of 100 cc., and analyzed for nitrogen and phosphorus, 1.0 cc. of the solution being used for each determination. The values found showed that 97.7 per cent of the nitrogen and 99.4 per cent of the phosphorus contained in the phospholipid were present in the aqueous solution. The balance of the solution, 98.0 cc., was freed quantitatively of sulfuric acid with barium hydroxide and the barium sulfate was removed by filtration. The filtrate was

concentrated *in vacuo* to about 55.0 cc., after which it was neutralized to phenolphthalein with barium hydroxide. A slight precipitate that separated consisted of barium phosphate and was removed by filtration. To the filtrate were added 2 volumes of alcohol, whereupon a white flocculent precipitate of barium glycerophosphate separated which was filtered off, washed with dilute alcohol, and with alcohol, and dried *in vacuo*.

The filtrate and washings were saved and examined for nitrogenous constituents.

*Barium Glycerophosphate*—The barium salt referred to above weighed 0.569 gm. It was twice precipitated from aqueous solution by addition of alcohol and was obtained as a snow-white amorphous powder that was easily soluble in water; it weighed 0.425 gm. For analysis the salt was dried to constant weight at 100° *in vacuo*.

<i>Analysis</i> — $C_3H_7O_6PBa(307.4)$ .	Calculated.	Ba 44.70, P 10.08
	Found.	" 44.41, " 9.65

The values found are in agreement with the calculated composition of barium glycerophosphate.

*Isolation of Choline As Chloroplatinate*—The filtrate from the barium glycerophosphate was acidified with hydrochloric acid and concentrated to dryness *in vacuo*. The residue was extracted three times with absolute alcohol. The extracts were combined and concentrated *in vacuo* to a volume of 25 cc. The addition of an alcoholic solution of chloroplatinic acid in slight excess gave a yellow precipitate which was filtered off, washed with absolute alcohol, and dried *in vacuo*. The substance weighed 0.529 gm. and after two recrystallizations from water by addition of alcohol 0.423 gm. of orange-colored crystals was obtained.

<i>Analysis</i> — $(C_5H_{14}ONCl)_2PtCl_4(616)$ .	Calculated,	Pt 31.68; found, Pt 31.60
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The filtrate from the choline chloroplatinate was evaporated to dryness. The residue was dissolved in water, acidified with hydrochloric acid, and the excess of platinum was removed with hydrogen sulfide, and filtered off. The filtrate on evaporation to dryness left a very small residue which was not further examined. The results obtained indicate that the principal nitrogen compound present in the phospholipid was choline.

*Examination of the Water-Insoluble Constituents*—The crude fatty acids obtained as mentioned previously were dissolved in 50 cc. of hot methyl alcohol and to the solution was added a hot saturated solution of barium hydroxide until the reaction was strongly alkaline. The mixture was heated to boiling, cooled, diluted with acetone, and the barium soaps were filtered off and washed with acetone. The filtrate and washings were evaporated to dryness *in vacuo* and the residue was extracted with hot acetone. The insoluble portion was added to the barium soaps and the latter were examined as will be described later.

The acetone extract on evaporation to dryness left a residue which weighed 55 mg. The substance was dissolved in 3 cc. of alcohol and the solution was carefully neutralized with dilute alcoholic sulfuric acid. The white precipitate which separated was filtered off, washed with cold alcohol, and recrystallized from a small volume of alcohol. The white crystals that were obtained weighed 11 mg. and apparently represented dihydrosphingosine acid sulfate.

*Analysis*— $C_{13}H_{25}O_2NH_2SO_4$ (399). Calculated, N 3.50; found, N (Kjeldahl) 3.45

*The Fatty Acids*—The fatty acids recovered from the barium soap weighed 1.144 gm., corresponding to about 69.8 per cent of the phospholipid. Since the phospholipid possessed a low iodine number, an attempt was made to separate the unsaturated from the saturated fatty acids by the lead soap-ether procedure. The ether-soluble lead soap yielded 0.105 gm. of fatty acids, while the acids recovered from the ether-insoluble lead soap weighed 0.999 gm.

The acid isolated from the ether-soluble lead soap was a semi-solid mass. The iodine number was 11.2 and the equivalent weight determined by titration was 472. This fraction was undoubtedly a mixture and owing to the small amount was not further examined.

The main fatty acid fraction obtained from the ether-insoluble lead soap was examined as follows: In order to remove any higher hydroxy acid such as phrenosinic acid, which might be a possible contaminant derived from cerebroside, the total crude acid was dissolved in 50 cc. of hot alcohol and a hot alcoholic solution of magnesium acetate was added carefully until no further precipita-

tion occurred. The precipitate was removed immediately by filtration and washed with hot alcohol, after which the free acid was recovered in the usual manner. The acid weighed only 29 mg. and its equivalent weight determined by titration was 396. Its properties resembled those of the phrenosinic acid isolated from the cerebroside fraction, but owing to the small amount available the acid could not be identified.

The acid recovered from the alcoholic solution, after the acid mentioned above had been precipitated as the magnesium salt, weighed 0.969 gm. and its equivalent weight was 273. This fraction was esterified with diazomethane, after which the methyl ester was fractionated twice through a column of the Craig (10) type. The principal ester fraction, 0.783 gm., m.p. 27–28°, corresponded to methyl palmitate. The distillation residues weighed 0.153 gm. and evidently consisted of a mixture of methyl palmitate and an ester of a higher acid, as shown below.

*Identification of Palmitic Acid*—The ester fraction corresponding to methyl palmitate gave on saponification an acid which crystallized in thin colorless plates. After two recrystallizations from 70 per cent methyl alcohol the acid melted at 62.5–64°. The molecular weight determined by titration was 256.

*Analysis*— $C_{16}H_{32}O_2$  (256). Calculated. C 75.00, H 12.50  
Found. " 74.92, " 12.45

The properties and composition of the acid indicate that it was pure palmitic acid.

The ester residues mentioned above were combined, saponified, and the free acid was isolated. The acid did not give the usual plate-shaped crystals characteristic of higher acids, but separated as an amorphous powder from 70 per cent methyl alcohol. After it had been precipitated in this manner four times, the substance was obtained as a white amorphous powder that weighed 68 mg. It melted at 76–78°, and the molecular weight determined by titration was 368.

*Analysis*— $C_{24}H_{48}O_2$  (368). Calculated. C 78.26, H 13.04  
Found. " 78.14, " 13.24

The values found for molecular weight and for carbon and hydrogen are in agreement with the calculated composition of a

tetracosanoic acid but the low melting point would indicate that the acid was not pure.

#### SUMMARY

The ether-insoluble fraction of the lipids obtained from *Cysticercus fasciolaris* larvae has been found to consist of a mixture of a saturated cerebroside and a hydrolecithin.

The cerebroside fraction was composed principally of dihydrophrenosin. After hydrolysis the following products were isolated, galactose, phrenosinic acid, a small amount of lower fatty acids, and a saturated nitrogenous base corresponding in composition to dihydrosphingosine.

The hydrolecithin was essentially dipalmitolecithin. The principal cleavage products obtained on hydrolysis consisted of palmitic acid, glycerophosphoric acid, and choline.

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## LETTERS TO THE EDITORS

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### ON THE NITROGENOUS CONSTITUENT OF CEPHALIN

*Sirs:*

Experiments carried out by the writer during 1940 clearly showed that ethanolamine could not be the only nitrogenous constituent of cephalin. As an attempt to isolate the unknown component from the hydrolysate of 25 gm. of cephalin was unsuccessful, it was thought proper to postpone publication until it was possible to repeat the isolation experiment on a somewhat greater scale. The evidence quite recently given by Folch and Schneider<sup>1</sup> of serine being a constituent of cephalin prompts me, however, to give a short account of my experiments.

In work on blood serum phosphatides already published<sup>2</sup> the writer found a ratio of 2:3 between phosphorus not bound to choline and ethanolamine nitrogen. This was partly referred to losses inherent in the method used for the determination of ethanolamine. The possible presence of another nitrogenous constituent than choline and ethanolamine was pointed out, and at the same time it was emphasized that the question whether cephalin contains nitrogen in another form than ethanolamine could not be regarded as settled.

In connection with these studies a series of ethanolamine determinations was performed on pure cephalin.<sup>3</sup> The analytical procedure used<sup>1</sup> is based on the volatility of ethanolamine at 75–80° at a pressure of about 10 mm. of Hg. With different preparations of cephalin, acid as well as alkaline hydrolysis of varying length of time, and with either ethanol or water as a solvent, in no instance was more than 25 to 50 per cent of the total nitrogen distilled as ethanolamine. It was further found that the fractions of cephalin least soluble in methanol contained less ethanolamine than fractions somewhat more soluble in this solvent. Moreover,

<sup>1</sup> Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).

<sup>2</sup> Blix, G., *Biochem. Z.*, **305**, 129 (1940).

<sup>3</sup> Prepared from ox brain according to Wadsworth, Maltaner, and Maltaner (*J. Immunol.*, **26**, 25 (1934)) and free from choline.



the cephalin nitrogen which after hydrolysis could not be distilled off at 10 mm. of Hg and 80° did not distil even if the temperature was raised to 160°. This last result seemed to exclude the possibility that the unknown component is a near homologue of ethanolamine or some other related amine, *e.g.* aminopropanediol, and, as all the nitrogen of cephalin is present as primary amino nitrogen, indicated that it may be a hydroxyamino acid, *e.g.* serine, which is closely related to ethanolamine.

My results are thus in good agreement with those of Folch and Schneider and suggest a possible way for the separation of ethanolamine cephalin from amino acid cephalin by means of an organic solvent.

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GUNNAR BLIX

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## THE ISOLATION OF LANTHIONINE FROM HUMAN HAIR, CHICKEN FEATHERS, AND LACTALBUMIN

*Sirs:*

In a recent publication<sup>1</sup> the authors described the isolation from wool of a new thio ether diamino acid, lanthionine,  $\text{HOOC}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$ . The wool was first boiled for 1 hour with 2 per cent  $\text{Na}_2\text{CO}_3$  solution and the treated fiber was then hydrolyzed with 20 per cent  $\text{HCl}$ . The isolation of this amino acid raised the question whether other proteins than wool would yield lanthionine under similar treatment. We are now able to report that we have isolated lanthionine not only from the keratins, human hair and chicken feathers, but also from lactalbumin by following the same procedure as was used in the case of wool. It therefore seems probable that lanthionine may be similarly obtained from most proteins which yield cystine on acid hydrolysis.

Another question of interest which presented itself was whether the reaction during pretreatment of the proteins with  $\text{Na}_2\text{CO}_3$  represented a general alkali effect or was due to some influence peculiar to  $\text{Na}_2\text{CO}_3$ . That it was due to alkali effect was shown by the isolation of lanthionine from wool that had been boiled for 1 hour with 0.1  $\text{N}$   $\text{NaOH}$  or with 2 per cent  $\text{Na}_2\text{S}$  previous to acid hydrolysis. It is of interest to note, however, that when higher concentrations of alkali were used, for example  $\text{N}$   $\text{NaOH}$  solution, all the wool was dispersed and hydrolysis of the products yielded cystine instead of lanthionine.

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<sup>1</sup> Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **138**, 141 (1941).



## ISOLATION OF A NUCLEOTIDE ESSENTIAL FOR THE GROWTH OF *LACTOBACILLUS CASEI*

Sirs:

Snell and Peterson<sup>1</sup> demonstrated the existence of a hitherto unrecognized growth factor for *Lactobacillus casei* which was distinct from riboflavin, pantothenic acid, nicotinic acid, and vitamin B<sub>6</sub>. On the basis of certain chemical properties these workers suggested that the growth factor may be a purine. This communication deals with the isolation and chemical properties of this growth factor.

The biological assay was carried out according to the technique described by Snell and Peterson.<sup>1</sup> The factor was isolated from solubilized liver by adsorption on norit followed by elution with 0.5 N NH<sub>4</sub>OH in 70 per cent methanol. Final purification was effected by fractionally precipitating the manganese salt of the factor with methanol. The most active preparation obtained by this method could not be further increased in activity by precipitation with heavy metals or by fractional precipitation from a concentrated aqueous solution.

The final product had the properties of a nucleotide in that it contained nitrogen, phosphorus, and gave a positive Bial's test for pentose. The pentose was not desoxyribose, as it gave a negative Feulgen test.<sup>2</sup> Approximately half of the phosphorus was hydrolyzed by 0.5 N H<sub>2</sub>SO<sub>4</sub> at 100° in 2 hours, while the remainder was removed more slowly. Only half of the theoretical ribose was obtained by hydrolysis with 0.5 N H<sub>2</sub>SO<sub>4</sub>. Equal equivalents of phosphoric acid and ribose were obtained during the first 2 hours of hydrolysis. As purine nucleotides are hydrolyzed rapidly and pyrimidine nucleotides more slowly, it appears that the active factor contains a purine and a pyrimidine nucleotide. This "dinucleotide," or mixture of nucleotides,

<sup>1</sup> Snell, E. E., and Peterson, W. H., *J. Bact.*, **36**, 273 (1940).

<sup>2</sup> Caspersson, T., *Biochem. Z.*, **253**, 97 (1932).

contains guanine but no adenine. The pyrimidine base has not yet been identified.

This "dinucleotide" may be partially replaced by a combination of guanine and thymine. However, the amounts required of these free bases are much greater than that of the liver "dinucleotide." Either the purine or the pyrimidine alone has little effect on growth. The maximum growth rate which can be obtained with an excess of thymine plus guanine is less than that produced by the factor isolated from liver. These results are shown in the accompanying table.

Supplement per 10 ml. medium	0.1 N acid produced per 10 ml. medium in 72 hrs. ml.
None.....	1.5
0.1 $\gamma$ liver "dinucleotide".....	4.7
0.2 $\gamma$ " ".....	7.6
0.5 $\gamma$ " ".....	10.2
20 $\gamma$ thymine .. . . . . .	2.0
20 $\gamma$ " + 25 $\gamma$ guanine chloride .. . . . .	4.4
20 $\gamma$ " + 50 $\gamma$ " " .. . . . .	5.6
20 $\gamma$ " + 100 $\gamma$ " " .. . . . .	5.2
50 $\gamma$ guanine chloride .. . . . .	1.7
50 $\gamma$ " " + 2 $\gamma$ thymine .. . . . .	4.7
50 $\gamma$ " " + 5 $\gamma$ " " .. . . . .	5.8
50 $\gamma$ " " + 10 $\gamma$ " " .. . . . .	5.6

Adenine, hypoxanthine, and xanthine were as effective as guanine as the purine component. Uracil or cytosine could not replace thymine.

Snell and Mitchell<sup>3</sup> have recently shown that both purine and pyrimidine bases are essential to or stimulate the growth of *Lactobacillus arabinosus*, *Lactobacillus pentosus*, and *Leuconostoc mesenteroides*. With these organisms uracil, cytosine, or thymine could serve as the pyrimidine base.

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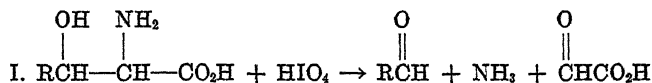
Received for publication, March 25, 1941

<sup>3</sup> Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **27**, 1 (1941).

## THE HYDROXYAMINO ACIDS OF SILK PROTEINS

*Sirs:*

The reaction<sup>1</sup> of periodic acid with  $\beta$ -hydroxy- $\alpha$ -amino acids according to Equation I has been shown to take place, under



suitable conditions, rapidly and quantitatively. And by the quantitative determination of the aldehydes formed, and of the ammonia liberated, rather good methods, valid for protein hydrolysates, have resulted for the estimation of serine, threonine,<sup>2</sup> and "total hydroxyamino acids."

These methods have been applied to a number of proteins, and the results will shortly be reported. But the results obtained on silk fibroin and silk sericin were so remarkable that immediate publication seemed desirable.

The proteins were obtained from Dr. Milton Harris of the Textile Foundation, National Bureau of Standards, and we wish to thank him for them. They were dried for some days in a vacuum desiccator before use. The results follow.

### *Balance Sheet for Hydroxyamino Acids in Silk Fibroin*

	Average serine equivalent per cent
Total hydroxyamino acids (calculated as serine), 15.0, 14.7	14.85
Serine found, 13.65, 13.7, 13.5, 13.45	13.57
Threonine found, 1.43, 1.50, 1.66, 1.62, 1.48	1.36
"Other" hydroxyamino acids	0.08

### *Balance Sheet for Hydroxyamino Acids in Silk Sericin*

Total hydroxyamino acids (calculated as serine), 43.8, 43.8	43.8
Serine found, 33.8, 34.0	33.9
Threonine found, 10.02, 10.16	8.9
"Other" hydroxyamino acids	1.0

<sup>1</sup> Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939).

<sup>2</sup> Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138**, 91 (1941).

We wish to make it clear that, so far as the results here reported go, *any part* of the "serine" reported could be hydroxylysine, in equimolecular proportion. And, in view of the rather large quantities involved, we prefer to interpret the low values for "other hydroxyamino acids" as indicating agreement rather than disagreement.

We do not know that any hydroxyamino acid other than serine has been reported as occurring in either of these proteins. For serine, the maximum figures reported (by the isolation method) have been 2.0 per cent for fibroin<sup>3</sup> and 6.81 per cent for sericin.<sup>4</sup>

Subject to confirmation by others, which we think will present no difficulty, we have thus established new high values for serine, threonine, and "total hydroxyamino acids" in any protein.

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<sup>3</sup> Abderhalden, E., and Spack, W., *Z. physiol. Chem.*, **62**, 131 (1909).

<sup>4</sup> Türk, W., *Z. physiol. Chem.*, **111**, 70 (1920).

## THE EFFECT OF VITAMIN A INTAKE ON VITAMIN A CONTENT OF BUTTER FAT

*Sirs:*

The carotene and vitamin A content of butter varies with the season and with the carotene intake.<sup>1</sup> However, the maximum levels obtainable after carotene are limited by its rate of absorption and speed of transformation to vitamin A. The use of cod liver oil to increase the vitamin A content of milk has been defeated by the decline in milk fat due to its toxicity.<sup>2</sup> As shark

Dietary procedure	No. of cows	Total vitamin A, i. u.					
		Basal diet; average of 4 wks.	Supplement period				
			2nd wk.	3rd wk.	9th wk.	15th wk.	19th wk.
Control; no supplement	6	44	41	35	36	55	31*
Supplement Group I, 700,000 i. u. throughout	3					72	
Supplement Group II,† 1,400,000 i. u. after 11th wk.	3	39	69	62	64	113	172* 170*

\* Value on one animal only.

† Average for animals on Supplement Groups I and II (six cows) through the 9th week.

liver oil, which is much higher in vitamin A and lower in vitamin D, offered promise, it was tested on twelve cows over a period of 5 to 7 months. During a preliminary basal period of 4 weeks, the basal level of vitamin A excretion was determined on the weekly samples. Analyses were made for carotene by the method of Koehn and Sherman<sup>3</sup> and for vitamin A with a Bills and Wallenmeyer photometer on butter fat obtained by churning pooled

<sup>1</sup> Baumann, C. A., and Steenbock, H., *J. Biol. Chem.*, **101**, 547 (1933).

<sup>2</sup> McCay, C. M., and Maynard, L. A., *J. Biol. Chem.*, **109**, 29 (1935).

<sup>3</sup> Koehn, C. J., and Sherman, W. C., *J. Biol. Chem.*, **132**, 527 (1940).



weekly samples of aliquots collected at each milking. After the preliminary period, Supplement Group I received 30 cc. of shark liver oil daily (700,000 i.u. of vitamin A) for the duration of the test (15 weeks), while Supplement Group II was fed the same dose for 10 weeks, followed by double the dose (1,400,000 i.u.) for the rest of the test (3 months with one cow). The accompanying table summarizes the results.

Bioassay on the samples from the 19th week gave values of 21 i.u. per gm. for the control and greater than 155 i.u. for the sample from the cow receiving 1,400,000 i.u. daily. No toxic symptoms were noted and the change in level of butter fat was similar in both groups. A considerable decrease in carotene excretion occurred in cows receiving the shark liver oil.

The milk production, which showed a gradual decline throughout the experiments owing to the lactation cycle, promptly rose in the animals receiving the vitamin A supplement and continued at a level approximately 10 per cent higher throughout the test. Likewise the level of butter fat rose slightly more after the vitamin A, the average increase per cow per week in the supplement group as compared with the control group being 599, 580, 507, and 794 gm. for the 2nd, 3rd, 9th, and 15th weeks respectively.

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## THE ISOLATION OF *l*-SERINE FROM SILK FIBROIN

*Sirs:*

The amino acid *l*-serine has been isolated from protein hydrolysates by several investigators; but in all cases the yields have been low, and the procedures impractical from a preparative standpoint. The method usually employed to obtain *l*-serine has therefore been the resolution of the *p*-nitrobenzoyl derivative of the synthetic *dl*-amino acid over the quinine and brucine salts, by the procedure of Fischer and Jacobs.<sup>1</sup>

While investigating, with the aid of aromatic sulfonic acids, the products of the hydrolysis of silk fibroin, we have isolated substantial amounts of *l*-serine as *p*-hydroxyazobenzene-*p'*-sulfonate. From this salt the free *l*-serine is readily obtained.

Technically degummed Japanese silk was hydrolyzed with concentrated hydrochloric acid and the mineral acid removed by means of vacuum distillation and lead acetate. After removal of free tyrosine, glycine was precipitated as 5-nitronaphthalene-1-sulfonate,<sup>2</sup> alanine as azobenzene-*p*-sulfonate, and the residual amounts of these sulfonic acids as barium salts. Addition of *p*-hydroxyazobenzene-*p'*-sulfonic acid precipitated *l*-serine hydroxyazobenzene sulfonate. The salt was recrystallized twice from water. The yield was 34 gm. of salt (equivalent to 9 gm. of *l*-serine) per 100 gm. of silk. Free *l*-serine was obtained by decomposition of the salt with barium acetate and the amino acid recrystallized once from water and alcohol. Yield, 75 per cent of the theory, calculated on the basis of the salt.

$C_8H_7O_3N$ . Calculated. C 34.3, H 6.7, N 13.3

Found. " 34.4, " 6.85, " 13.4

$[\alpha]_D^{25} = +13.85^\circ$ , 10% in *N* HCl;  $[\alpha]_D^{25} = -6.70^\circ$ , 10% in water

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<sup>1</sup> Fischer, E., and Jacobs, W. A., *Ber. chem. Ges.*, **39**, 2942 (1906).

<sup>2</sup> Doherty, D. G., Stein, W. H., and Bergmann, M., *J. Biol. Chem.*, **135**, 487 (1940).

Fischer and Jacobs<sup>1</sup> reported  $[\alpha]_D^{28} = +14.45^\circ$  and  $[\alpha]_D = -6.83^\circ$ .

Although the yield of *l*-serine obtained in the above experiment exceeds any reported in the literature, it does not, of course, represent the total amount of serine present in a silk fibroin hydrolysate. We have estimated the amount of ammonia liberated from a fibroin hydrolysate when treated with periodate according to the method of Nicolet and Shinn<sup>3</sup> and Van Slyke *et al.*<sup>4</sup> It was found that 12 per cent of the nitrogen of silk fibroin is hydroxyamino acid nitrogen.

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<sup>3</sup> Nicolet, B. H., and Shinn, L. A., *J. Am. Chem. Soc.*, **61**, 1615 (1939).

<sup>4</sup> Van Slyke, D. D., Hiller, A., MacFadyen, D. A., Hastings, A. B., and Klemperer, F. W., *J. Biol. Chem.*, **133**, 287 (1940).

## MECHANISM OF FIXATION OF CARBON DIOXIDE IN THE KREBS CYCLE

Sirs:

Oxidation of pyruvate by the Krebs cycle involves its union with oxalacetate. Liver in contrast to certain other tissue oxidizes pyruvate in the absence of added C<sub>4</sub>-dicarboxylic acid. It was proposed <sup>1, 2</sup> that the C<sub>4</sub>-dicarboxylic acid is synthesized in liver by the reaction of Wood and Werkman,<sup>3</sup>  $C^{13}O_2 + CH_3 \cdot CO \cdot COOH = C^{13}OOH \cdot CH_2 \cdot CO \cdot COOH$ . The isolation<sup>1</sup> of radioactive  $\alpha$ -ketoglutarate, formed from pyruvate in the presence of radioactive sodium bicarbonate, proved that there is fixation of carbon by liver.

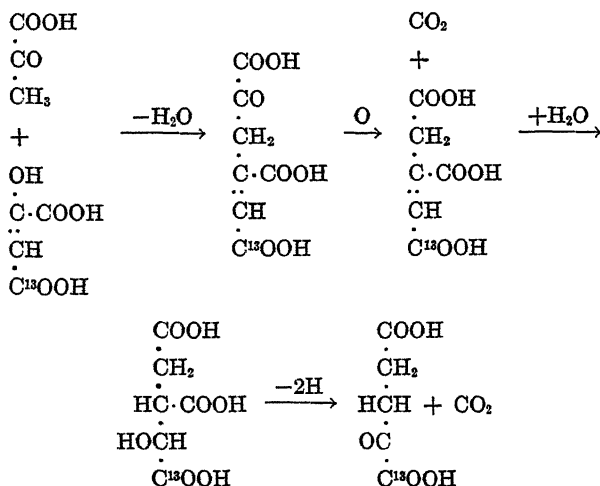
We have determined the location of the fixed carbon in  $\alpha$ -ketoglutarate by use of C<sup>13</sup>O<sub>2</sub>. The experimental procedure for the dissimilation of pyruvate was that employed by Evans and Slotin<sup>1</sup> except that sodium bicarbonate containing 9 per cent C<sup>13</sup> was used. 30 ml. of medium were used in each of six 125 ml. Warburg flasks. The  $\alpha$ -ketoglutarate was isolated from the deproteinated solution by binding it with bisulfite, saturating the solution with magnesium sulfate, and removing the citric, malic, succinic, and fumaric acids by continuous ether extraction for 3 days. The residue of extraction was freed from sulfite by boiling, extracted with ether for 24 hours, and the  $\alpha$ -ketoglutarate was separated from the pyruvate by precipitation as the silver salt. The free acid was obtained by acid ether extraction. The titratable acidity was equivalent to 0.32 mm and the 2,4-dinitrophenylhydrazone (m.p. 223°, mixed m.p. 223°) 0.28 mm of  $\alpha$ -ketoglutarate. The acid was oxidized with potassium permanganate to CO<sub>2</sub> and succinic acid,  $C^{13}OOH \cdot CO \cdot CH_2CH_2 \cdot COOH = C^{13}O_2 + COOH \cdot CH_2 \cdot CH_2 \cdot COOH$ . The C<sup>13</sup> content of the CO<sub>2</sub> and succinate was determined with the mass spectrometer. Succinate

<sup>1</sup> Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 301 (1940).

<sup>2</sup> Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 1383 (1940).

<sup>3</sup> Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **139**, 365, 377 (1941).

contained the natural complement of  $C^{13}$  (1.10 per cent), whereas the  $CO_2$  contained a high concentration of  $C^{13}$  (2.20 per cent). These results prove that all the fixed carbon is in one carboxyl group of the  $\alpha$ -ketoglutarate. A possible mechanism of formation of this acid is shown in the accompanying scheme.



The position of the fixed  $C^{13}$  in the  $\alpha$ -ketoglutarate is in agreement with its derivation from pyruvate and oxalacetate, the initial fixation of  $CO_2$  being in the oxalacetate. The results clearly indicate that citrate is not an intermediate, for, if  $\alpha$ -ketoglutarate was derived from a symmetrical molecule, the fixed carbon would be equally distributed in the two carboxyl groups.

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## INOSITOL: A LIPOTROPIC FACTOR

*Sirs:*

It was reported by us last year that feeding a beef liver fraction to rats causes the production of acutely fatty livers containing large amounts of cholesterol. The production of these fatty livers is only slightly affected by giving choline but is completely prevented by the simultaneous administration of lipocaic. Using a procedure similar to that for the preparation of lipocaic, we have prepared extracts from liver, pancreas, kidney, muscle, wheat germ, yeast, and rice polishings and have found that these are all effective in preventing this type of fatty liver in rats. It has been reported by us that the same kind of fatty liver can be produced by supplying rats with biotin in conjunction with thiamine, riboflavin, pantothenic acid, and pyridoxine.

In studies on the prevention of the "biotin" type of fatty liver in rats we have fed inositol. This substance prevents the development of the acutely fatty liver and the accumulation of cholesterol in the liver. The prevention of the fatty liver has been secured in three series of animals, in each of which groups of ten rats have been employed. In these experiments on rats the action of inositol thus resembles that of lipocaic.

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## CYSTINURIA

### THE METABOLISM OF CYSTINE, CYSTEINE, METHIONINE, HOMOCYSTEINE, AND S-CARBOXYMETHYLCYSTEINE

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Although it is believed that the urinary cystine in cystinuria is produced by the metabolism of certain dietary constituents (1, 2), agreement seems to be less general regarding the nature of the intermediary changes involved. Kassell and coworkers (3) have shown that extra cystine was excreted when methionine, cysteine, and homocysteine were fed to a cystinuric, but not when cystine, homocysteine, and glutathione were administered. By feeding casein, egg albumin, and lactalbumin, it was shown that cystine and methionine in combined form were catabolized by the cystinuric in the same way as when they were free. When lactalbumin and reduced lactalbumin were fed, it was shown that more extra cystine was produced by the latter, and, since reduced lactalbumin contains much more cysteine (and no more methionine) than lactalbumin, it was concluded that, "Cystine and cysteine as part of the same protein molecule behave as two different amino acids with separate catabolic pathways."

Brand, Cahill, and Kassell (4) have confirmed the observation of Lewis, Brown, and White (5) that, when the cystinuric is on a high protein diet, less extra cystine is excreted after methionine administration than when he is on a diet lower in protein. In a preadolescent cystinuric boy, Andrews and Randall (6) observed no significant amounts of extra cystine, after feeding large quantities of methionine, even though the subject was maintained on a moderate protein diet. 3 years later Andrews and Andrews (2) found that, in the same cystinuric subject, methionine administration gave rise to a 30 per cent increase in cystine excretion.



In view of the conflicting results cited above, it seemed desirable to extend the data by a study of an additional case. In the experiments reported here it was found that this subject, on a rather high protein diet, produced no extra cystine after ingestion of cystine and homocystine, that extra cystine was obtained after administration of cysteine, but that no extra cystine appeared after methionine was fed unless the level of protein in the diet was lower.

Furthermore, the results obtained after feeding this subject S-carboxymethylcysteine did not support the suggestion of Brand, Block, Kassell, and Cahill (7) that this substance may be of therapeutic value in cystinuria.

#### EXPERIMENTAL

*Case History*—The subject of these experiments was a young woman, 27 years old, who weighed 45 kilos. The experimental study was made during one metabolic period in 1938 and another in 1939. In 1935 the subject's right kidney was removed surgically, because of renal colic which was associated with x-ray evidence of the presence of stones. Repeated microscopic examination of sediments obtained by centrifuging the urine of this subject revealed on some days a very few crystals which resembled those of cystine, but ordinarily no such crystals were observed.

*Diet*—For the 1939 series, diets of variable composition were arranged in such a way that they were nearly identical with respect to the amount of total protein, of protein high in sulfur content, and of total calories. The variation of total protein was between 80 and 92 gm. (average, 84 gm.), of protein high in sulfur content it was between 38 and 41 gm. (average, 39 gm.), and of total calories between 2200 and 2700 (average, 2400). Five diets were provided in order to avoid monotony. The subject was of a high degree of intelligence and, although she lived at home and prepared and measured her own food, she excreted the various urinary constituents at a constant level during the control periods; this indicated that she performed her share of the work in a satisfactory manner. In the 1938 series protein was fed at a lower level. See "Discussion."

*Preparation of Sulfur Compounds*—S-Carboxymethylcysteine was prepared from cysteine hydrochloride and monochloroacetic

acid by the method of Michaelis and Schubert (8). The product had the following composition.

$C_5H_9O_4NS$ . Calculated, N 7.82, S 17.88; found, N 7.79, S 17.86

Michaelis and Schubert reported that their product melted with decomposition at 175–176°. Our product, after several recrystallizations, melted with decomposition at 193–194°.¹ When tested quantitatively in the Sullivan-Hess procedure, this product seemed to contain a small amount of cystine as an impurity.

Homocystine was prepared by the method of Patterson and du Vigneaud (9), the alcohol process being used for the reduction of S-benzylhomocystine. The product had the following composition.

$C_8H_{16}O_4N_2S_2$ . Calculated. N 10.44, S 23.90  
Found. " 10.17, " 23.75

Cystine of satisfactory purity was prepared from human hair by the usual method.

Cysteine hydrochloride and methionine (c.p. grade) were obtained from the Eastman Kodak Company. These compounds were assumed to be of satisfactory purity.

*Methods*—The 24 hour samples of urine were preserved under toluene. In addition to the urinary constituents recorded in Table I, pH was estimated colorimetrically, and creatinine by the colorimetric method of Folin. Procedures for the estimation of the recorded constituents were as follows: total nitrogen, Kjeldahl; total sulfur, gravimetric (Benedict-Denis); total and inorganic sulfates, gravimetric (Folin); cystine, a modification of the method of Sullivan and Hess (10). All of the compounds were ingested in gelatin capsules and were fed, except in the case of S-carboxymethylcysteine, in such quantities that each day's intake corresponded in sulfur content to that of 2 gm. of cystine. The 12 gm. of S-carboxymethylcysteine fed during 3 days contained an amount of sulfur corresponding to that in slightly more than 8 gm. of cystine.

¹ Dr. F. R. Blood, of the University of Michigan, measured the melting points (all corrected) of three different samples of S-carboxymethylcysteine. A sample obtained from Dr. E. Brand melted at 191–192°. A sample prepared by Dr. Blood, by a modification of the method of Michaelis and Schubert, melted at 191–192°. Our sample melted at 193–194°.

Like others who have used the Sullivan method, we have encountered some difficulty with it and have introduced minor changes in order to obtain reproducible values. Andrews and Andrews (11) have shown that fresh urine may contain ascorbic acid and other reducing substances which produce low results in the original Sullivan method, and that, although the modification of Sullivan and Hess (10) minimizes this interference, it does not completely remove it. Since the subject of the present study lived 30 miles from the laboratory, the urine sample completed on a given morning was not analyzed until the next day at the earliest. A portion of the day's sample was aerated for 3 hours by drawing through it room air which passed first through water at a temperature the same as that of the urine. That these conditions and this preliminary aeration served to remove the inhibitory substances was indicated by the constancy of the cystine values after further aeration. It is possible, of course, that some inhibitors which resisted oxidation during the 3 hours of aeration remained even after this prolonged treatment. Examination of the data in Table I will show, however, that the level of urinary cystine was reasonably uniform on the days when the basic diet only was ingested.

Despite the use of several samples of sodium hydrosulfite, we were unable to convert the brown, turbid mixture to a clear, cherry-red solution, by using 1 ml. of a 2 per cent solution of sodium hydrosulfite in 0.5 N sodium hydroxide. This difficulty was overcome by using 3 ml. of the sodium hydrosulfite solution.

*Metabolic Observations*—As can be seen from the data presented in Table I, on the days the basic diet was fed alone the urinary constituents were excreted at a constant level, except for a somewhat erratic excretion on those days following the administration of S-carboxymethylcysteine. Although the values are not presented in Table I, the creatinine excretion was constant, which indicated that complete 24 hour specimens were collected throughout the study. In the 1939 series 3 gm. of "citrocarbonate" were ingested each day, except that 4.5 gm. were eaten on each of the 2 days on which cysteine hydrochloride was fed, and on the days when 2, 4, and 6 gm. of S-carboxymethylcysteine were administered, 4, 5, and 6 gm., respectively, of "citrocarbonate" were used. Under these conditions the pH varied only between 6.8 and 7.2 on all but 2 days; on 1 day it was 6.6 and on another, 6.7.

TABLE I  
*Metabolic Observations in Cystinuria*

Period No.	Date	Substance fed (gm.)	Volume	Total N	Total S	Total sulfate S	Organic S	Cystine S	Undetermined organic S	Cystine
	1933		ml.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	Mar. 7*	Cystine, 2.00	1610	7.5	0.57	0.34	0.23	0.17	0.06	0.64
	" 8*		1910	7.2	0.55	0.31	0.24	0.17	0.07	0.65
2	" 9		2475	7.9	0.99	0.76	0.23	0.17	0.06	0.62
	" 10		2440	8.4	0.96	0.73	0.23	0.14	0.09	0.53
	" 11		1565	8.4	0.69	0.46	0.23	0.15	0.08	0.57
	" 12	Methionine, 2.48	1560	8.2	0.62	0.39	0.23	0.18	0.05	0.66
3	" 13*		1760	8.7	0.58	0.35	0.23	0.16	0.07	0.60
4	" 16†		1800	8.4	0.64	0.37	0.27	0.19	0.08	0.70
	" 17†		2240	8.1	0.56	0.31	0.25	0.19	0.06	0.70
5	" 18		1740	7.5	0.88	0.54	0.34	0.24	0.10	0.90
	" 19	" 2.48	1585	8.0	1.15	0.78	0.37	0.23	0.14	0.86
6	" 20†		1410	6.7	0.56	0.32	0.24	0.19	0.05	0.71
	" 21†		1877	8.5	0.61	0.37	0.24	0.20	0.04	0.76
	" 22†		1635	7.4	0.57	0.35	0.22	0.19	0.03	0.71
	1939									
7	Feb. 24†	Cysteine hydrochloride, 2.63	2190	9.3	0.77	0.52	0.25	0.18	0.07	0.66
	" 25†		1545	10.0	0.71	0.44	0.27	0.17	0.10	0.64
	" 26†		1885	10.0	0.71	0.46	0.25	0.18	0.07	0.68
	" 27†		1340	9.6	0.64	0.39	0.25	0.17	0.08	0.65
	" 28†		1865	9.9	0.70	0.44	0.26	0.17	0.09	0.62
8	Mar. 1	Cysteine hydrochloride, 2.63	1748	10.4	1.00	0.70	0.30	0.21	0.09	0.79
	" 2		1845	9.8	1.26	0.97	0.29	0.21	0.08	0.78
	" 3		1450	10.9	0.80	0.53	0.27	0.18	0.09	0.68
9	" 4†	Carboxymethylcysteine, 2.00	1595	10.6	0.77	0.50	0.27	0.17	0.10	0.63
	" 5†		2275	10.5	0.76	0.51	0.25	0.17	0.08	0.63
	" 6†		1650	10.4	0.79	0.54	0.25	0.16	0.09	0.59
10	" 7		2220	10.5	1.09	0.62	0.47	0.13	0.34	0.50
	" 8	Carboxymethylcysteine, 4.00	1840	10.1	1.30	0.65	0.65	0.12	0.53	0.46
	" 9	Carboxymethylcysteine, 6.00	2440	12.6	1.65	0.78	0.87	0.12	0.75	0.46
	" 10		2330	10.9	1.09	0.69	0.40	0.14	0.26	0.51
11	" 11†		1485	10.0	0.77	0.49	0.28	0.17	0.11	0.64
	" 12†		1940	9.6	0.86	0.58	0.28	0.18	0.10	0.67
	" 13†		1580	10.5	0.82	0.56	0.26	0.18	0.08	0.66
	" 14†		1940	9.9	0.66	0.39	0.27	0.17	0.10	0.63

TABLE I—*Concluded*

Period No.	Date	Substance fed (gm.)	Volume	Total N	Total S	Total sulfate S	Organic S	Cystine S	Undetermined organic S	Cystine
	1939		ml.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
12	Mar. 15	Homocystine, 2.23	2060	8.5	0.84	0.57	0.27	0.18	0.09	0.67
	" 16	" 2.23	2180	9.6	1.06	0.82	0.24	0.17	0.07	0.65
	" 17		2445	9.5	0.87	0.61	0.26	0.17	0.09	0.62
13	" 18§	No evening meal	1160	8.0	0.60	0.36	0.24	0.16	0.08	0.61
14	Apr. 5†		1710	7.7	0.57	0.35	0.22	0.16	0.06	0.61
	" 6†		1550	9.0	0.58	0.36	0.22	0.16	0.06	0.61
15	" 7	Methionine, 2.48	2160	9.9	1.16	0.84	0.32	0.18	0.14	0.68
	" 8	" 2.48	1860	9.7	1.27	0.94	0.33	0.17	0.16	0.64
	" 9		1970	9.6	0.91	0.64	0.27	0.17	0.10	0.63
16	" 10†		1140	10.0	0.70	0.48	0.22	0.17	0.05	0.63
	" 11†		1370	10.0	0.63	0.40	0.23	0.17	0.06	0.63
	" 12†		2080	9.1	0.71	0.46	0.25	0.17	0.08	0.65
	" 13†		1760	8.9	0.62	0.39	0.23	0.17	0.06	0.63
Average, Periods 4 and 6.....					0.59	0.34	0.24	0.19	0.05	0.72
" " 7, 9, 11, 14, 16.....					0.71	0.46	0.25	0.17	0.08	0.64

\* These values used in calculation of average values for control Periods 1 and 3 (these averages not listed here).

† These values used in calculation of average values for control Periods 4 and 6.

‡ These values used in calculation of average values for control Periods 7, 9, 11, 14, and 16.

§ Experiments interrupted because the subject contracted influenza.

*Cystine*—This subject oxidized the sulfur of cystine as readily as those subjects studied by others. No extra cystine appeared after 4 gm. of cystine were fed over 2 days (Table I, Period 2, 1938 series) and all of the extra sulfur appeared as sulfate (Table II).

*Cysteine*—Administration of 5.26 gm. of cysteine hydrochloride over 2 days (Table I, Period 8) resulted in the excretion of 0.33 gm. of extra cystine. No extra undetermined organic sulfur was found. A considerable portion of the cysteine sulfur was oxidized to sulfate (77 per cent, Table II).

*Methionine*—After ingesting 4.96 gm. of *dl*-methionine this

TABLE II  
*Extra Sulfur and Extra Cystine in Experimental Periods*

Period No.*	Substance fed	Extra sulfur												Extra cystine gm.
		Fed	Excreted as								Cystine S			
			Total S		Sulfate S		Organic S		Undetermined organic S					
gm.	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent	gm.	per cent		
2	Cystine	1.07	0.98	92	1.02	95	0.00		0.00		-0.03‡		-0.14‡	
8	Cystine hydrochloride	1.07	0.93	87	0.82	77	0.11	10	0.02	2	0.09	8	0.33	
5	Methionine§	1.07	0.85	79	0.64	60	0.23	21	0.14	13	0.09	8	0.32	
15	Methionine	1.07	1.21	113	1.04	97	0.17	16	0.16	15	0.01	0.9	0.03	
12	Homocystine	1.07	0.64	60	0.62	58	0.02	2	0.01	0.9	0.01	0.9	0.02	
10	Carboxymethylcystine	2.15	2.29	107	0.90	42	1.39	65	1.56	73	-0.17‡		-0.63‡	

\* All experimental periods consisted of 3 days, except Period 5, which consisted of 2 days, and Periods 2 and 10, which consisted of 4 days.

† Percentages of sulfur fed.

‡ Decreased excretion.

§ Low protein diet.

|| High protein diet.

subject excreted no extra cystine (Table I, Period 15), although the rise in undetermined organic sulfur may have been significant. The total sulfur excreted represented 113 per cent of the sulfur fed (Table II). This high recovery of sulfur is similar to that obtained by Lewis, Brown, and White (5). In two of the three experiments in which they administered methionine to their subject, when he was on a high protein diet, they recovered 107.6 and 110.9 per cent, respectively, of the sulfur fed as methionine. Further similarity in these two cystinuric subjects is seen in their apparent ability, when the basic diet was high in protein, to excrete nearly as much extra sulfur as sulfate as was fed in the form of methionine.

*Homocystine*—No extra cystine was excreted by our subject after the ingestion of 4.46 gm. of homocystine. Of the sulfur fed as homocystine, 60 per cent was recovered and all of this was in the oxidized fraction (Table II). Although the amount of sulfur recovered in this experiment was greater than that recovered by Brand, Cahill, and Block ((12) Table II), no extra organic sulfur was excreted; hence it would appear that, unlike their subject, our patient excreted no homocystine as such.

*S-Carboxymethylcysteine*—Following the administration of 12 gm. of S-carboxymethylcysteine over 3 days (Table I), 107 per cent of the sulfur fed was recovered as extra total sulfur, while 42 per cent was recovered as extra sulfate (Table II), and a pronounced increase in undetermined organic sulfur occurred. Cystine excretion, as measured by the Sullivan-Hess procedure (10), was depressed significantly. In Period 10 (Table I) the cystine excreted was 0.63 gm. less than the expected amount, as based on the average for the control periods (Table II). Neither the untreated urine, nor that which had been reduced with zinc and hydrochloric acid, yielded the odor of thioglycolic acid. Extraction of the reduced urine with ether and removal of the ether at room temperature failed to yield any residue with an odor of thioglycolic acid. Cystine crystals were not found in the urine of the 3rd day of S-carboxymethylcysteine administration, but they appeared to be present to a greater extent than usual in the urine of the first 2 days. By the use of acetic acid and heat a slight amount of protein was detected in the urine of the 3rd day, whereas no protein was found on days preceding or succeeding this. On

this day also the nitrogen excretion was considerably greater than would have been expected, even if all of the nitrogen in the 12 gm. of S-carboxymethylcysteine fed over 3 days were excreted on this 3rd day. During the 4 days (Period 11) immediately following this experiment the excretion of oxidized sulfur was somewhat higher than in other rest periods and it was rather erratic.

#### DISCUSSION

The failure of our subject to excrete extra cystine after ingesting cystine is in agreement with the results of similar experiments in other laboratories. The response of our cystinuric subject to homocystine ingestion was similar to that of the subject studied by Brand, Cahill, and Block (12), except for the difference that in our studies, as measured by changes in the organic sulfur, no excretion of unchanged homocystine, after administration of the latter, was observed.

The results of this study lend support to those of Brand, Cahill, and Harris (13) and to those of Lewis, Brown, and White (5), in that the feeding of cysteine to our subject produced extra cystine in the urine.

After feeding methionine, however, in the 1939 experiments, we were unable to find an increase in cystine excretion. We do not feel that the 0.68 gm. of cystine, obtained on April 7, 1939, is significantly higher than the average of 0.64 gm. per day for the control periods of this series. Although this result would seem to correspond with that obtained by Andrews and Randall (6), who found no extra cystine after feeding methionine to a preadolescent cystinuric boy, we are inclined toward the view that it confirms instead the findings of Lewis, Brown, and White (5), who obtained less extra cystine after methionine was fed to a cystinuric subject on a high protein diet than when the subject was on a low protein diet. From the data given by Lewis and coworkers, it would appear that their high protein diet, on which they obtained the least extra cystine following methionine ingestion, was fed at a level of 1.97 gm. per kilo. Our subject received protein at nearly the same level; namely, 1.87 gm. per kilo. One has only to assume a reasonable variation between the two cystinuric subjects to feel justified in predicting that, on a lower protein diet, our subject would have excreted extra cystine after



methionine administration. Such, indeed, was the case. In an experiment carried out in 1938 (Table I, Periods 4 to 6), at a level of about 1.2 gm. of protein per kilo, after ingesting the same amount of methionine as was given in the 1939 series (4.96 gm.), our subject excreted 0.32 gm. of extra cystine. It would seem, therefore, that the effect of variations in the level of dietary protein, observed in Lewis' laboratory, is more pronounced in some cystinuric subjects than in others and that sufficient variation in the level at which protein is fed might reveal similar behavior on the part of all cystinuric patients.

While the subject of our experiments did excrete less cystine after ingesting S-carboxymethylcysteine than was done in the control periods, the level of urinary cystine was not decreased nearly as much as in the cystinuric patients studied by Brand, Block, Kassell, and Cahill (7). Whereas these workers found a reduction in cystine excretion from 1 gm. to 200 mg. per day (an 80 per cent decrease), our subject's urinary cystine fell less spectacularly from about 0.64 gm. to about 0.48 gm. (a 25 per cent decrease). Cystine crystals did disappear from the urine of our subject, but only on the 3rd day of administration of S-carboxymethylcysteine. In this respect it would seem that our subject exhibited behavior intermediate to that exhibited by the two patients of Brand and coworkers, for crystals disappeared from the urine of only one of those (7). Our results do not seem to support the hypothetical explanation offered by these workers for the decrease in urinary cystine as measured by the method of Sullivan. We failed to detect any thioglycolic acid. To be sure we used the modification of the Sullivan and Hess (10) method, while Brand and coworkers used the Sullivan and the Lugg-Sullivan methods, but unless this difference in procedure is significant, we prefer another interpretation. In view of what is known about the Sullivan reaction, it would seem that any cystine present in the postulated "mixed disulfide" ((7) p. 502) would be reduced by cyanide and the cysteine so formed would react in the usual manner. The apparent reduction in urinary cystine was not caused by the presence in the urine of S-carboxymethylcysteine, excreted as such. For, in experiments<sup>2</sup> in which cystine

<sup>2</sup> These experiments were carried on in the laboratory of the Department of Biological Chemistry, University of Michigan.

and S-carboxymethylcysteine were added to normal urine, the presence of the latter compound did not interfere with the estimation of the cystine. This metabolic phenomenon should be examined further to learn whether the urine contains products arising from the metabolism of S-carboxymethylcysteine which interfere with the Sullivan reaction. In view of the failure of our subject to experience a marked diminution of cystine excretion, even after ingesting large quantities of S-carboxymethylcysteine, as well as the suggestion that it was mildly toxic in this case, we feel that its use as a therapeutic agent in cystinuria must be approached with great caution.

The subject of these experiments did not excrete increased amounts of cystine as a result of diuresis, as was done by the cystinuric subject studied by Andrews and coworkers (2, 6). Although the volumes of the 24 hour excretions varied markedly from day to day, no relationship between these volumes and the amount of cystine excreted is indicated by the data in Table I.

We wish to express our sincere appreciation to Dr. Lawrence Parsons, of St. Mary's Hospital, Reno, who referred the subject to us; to Dr. H. B. Lewis, from whom we have obtained valued encouragement and counsel; to the subject of these experiments whose splendid cooperation made them possible; and to Clyde Beck, Kirby Stoddard, and Frank Hickey, who contributed to the study in its early stages.

#### SUMMARY

1. Cystine and homocystine, when fed to a cystinuric woman, yielded no extra cystine in the urine. Cysteine hydrochloride, in a similar experiment, did produce extra cystine in the urine.

2. When *dl*-methionine was fed, no measurable increase in urinary cystine was observed when the patient was on a diet yielding 1.87 gm. of protein per kilo, but when a diet of lower protein content (1.2 gm. per kilo.) was fed, superimposition of *dl*-methionine did cause excretion of some extra cystine.

3. Administration of a total of 12 gm. of S-carboxymethylcysteine over a period of 3 days was followed by a reduced cystine excretion of approximately 25 per cent and by a disappearance of cystine crystals on the 3rd day only.

4. There was no indication that diuresis resulted in an increase in cystine excretion.

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## A BACTERIAL ASSAY METHOD FOR NICOTINAMIDE AND RELATED SUBSTANCES IN BLOOD, URINE, AND SPINAL FLUID

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Quantitative bacterial assays for nicotinamide and related substances have been reported by other workers. Following the demonstration by Knight (1) in 1937 that nicotinic acid was an essential factor for the growth of *Staphylococcus aureus* in synthetic media, Koser (2) and his associates in 1938 showed that many strains of dysentery bacilli required nicotinic acid for growth in amino acid mixtures, and Fildes (3), in the same year, found that nicotinic acid was essential for the development of certain strains of *Proteus* in a simple ammonium lactate medium. In 1938 Lwoff and Querido (4) reported that the amount of growth in a modification of Fildes' ammonium medium was, within limits, proportional to the amount of nicotinamide in the medium. Fraser, Topping, and Sebrell (5) in the same year reported an assay of urine for nicotinic acid and related substance, using the growth of *Shigella paradysenteriae* (Sonne). In 1939 Querido, Lwoff, and Lataste (6) used *Proteus* for the estimation of nicotinamide in blood and Lataste (7) adapted the *Proteus* test to the assay of nicotinamide in urine. Dorfman, Horwitt, *et al.* (8) in 1939 reported the quantitative determination of nicotinic acid in blood, milk, urine, and saliva and later (9) described the details of the method as applied to nicotinamide in blood.

It is the purpose of the present communication to present the details of a quantitative assay method for nicotinamide and related substances in biological fluids with use of *Shigella paradysenteriae* (Sonne) as the test organism. Some use is made of

the features of previously reported techniques but the method differs from them in that new methods of preparation of samples for assay have been developed which permit the rapid handling of large numbers of samples at one time.

*Stock Culture*—A strain of *Shigella paradyserteriae* (Sonne, N. I. H. 741) carried on beef infusion agar slants is used. Transfers are made at least bimonthly. On the day prior to an assay several fresh slants are inoculated from the stock culture and incubated at 31° for 24 hours. If desired, cultures may be transferred 6 hours before use and incubated at 37°.

*Basal Medium*—The medium used is based on the modification of the ammonium lactate medium of Fildes (3) developed by Lwoff and Querido (4) for the growth of *Proteus*. Growth of our strain of *Shigella paradyserteriae* is as good in this medium as in the amino acid mixture used by Fraser, Topping, and Sebrell (5).

The medium has the following composition:  $\text{KH}_2\text{PO}_4$ , anhydrous, 10.0 gm.,  $(\text{NH}_4)_2\text{SO}_4$  1.5 gm., KCl 1.0 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 gm., thiamine hydrochloride 0.02 gm., glucose 10.0 gm.,  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ , 0.01 M in 0.01 N HCl, 50 ml.; distilled water to make 1000 ml.

The medium is adjusted to pH 7.4 to 7.6 with sodium hydroxide before it is diluted to volume. 5 ml. of the medium are used in each assay tube.

The medium becomes turbid after standing for several days and, therefore, should be prepared on the day of assay. For this reason it is convenient to keep a concentrated stock solution of all the constituents except the glucose and ferrous ammonium sulfate. The medium may then be prepared by adding calculated amounts of dry glucose and fresh ferrous ammonium sulfate solution to the proper amount of the stock solution, the pH being adjusted to 7.4 to 7.6 and the medium diluted to volume.

*Standard Nicotinamide Solution*—A solution containing 0.05  $\gamma$  of nicotinamide per ml. is prepared on the day of assay. This solution must be freshly made, as we have found that dilute aqueous solutions of nicotinamide deteriorate rapidly unless precautions are taken to keep them sterile. Stock solutions of nicotinamide containing 100  $\gamma$  per ml. or more are stable for at least 5 days, but should be discarded after that time unless precautions are taken to keep them sterile.

*Procedure for Assay*

The assays are carried out in  $20 \times 175$  mm. test-tubes calibrated for use in the photoelectric photometer. We have found that the organisms multiply more rapidly (as judged by the turbidity produced) in these tubes than in the usual  $16 \times 150$  mm. test-tubes. A set of standards must be prepared with each set of assay because of differences in the response of the organisms from day to day. Thirteen tubes are used for the standards. Nicotinamide is not added to three tubes. Two of these are not inoculated and are used to balance the photometer. The third is an inoculated control. 1 to 5 ml. of the 0.05  $\gamma$  per ml. nicotinamide standard is pipetted into five pairs of tubes. 1 to 5 ml. of diluted blood, urine, or other material prepared as described below is pipetted into other tubes. Three to five tubes are used for each dilution of a material of unknown nicotinamide content. 5 ml. of the medium are added to each tube and, when necessary, the volume is brought up to 10 ml. by the addition of distilled water. All tubes are plugged with cotton and autoclaved for 15 minutes at 15 pounds pressure. The tubes should be kept well away from the sides of the autoclave and should not be autoclaved more than 15 minutes, since overheating results in brownish discoloration of the medium. After the tubes have cooled, each is inoculated (except the two uninoculated controls) with 1 drop of a barely visible suspension in sterile 0.85 per cent saline solution of the culture of *Shigella paradysenteriae*. The tubes are then incubated for 16 to 22 hours at  $31^{\circ}$  and the turbidity read in a photoelectric photometer.<sup>1</sup> Duplicates should check within 2 to 3 per cent.

The data obtained from the nicotinamide standard tubes are used to construct a curve on semilogarithmic graph paper, from which the response elicited by an unknown sample is evaluated by interpolation from the curve and a value is calculated in terms of "microgram equivalents to nicotinamide." The term "equivalents to nicotinamide" is used because the organism responds not only to nicotinamide but also nicotinic acid, nicotinuric acid, and a number of other related compounds (9-11), so that the value obtained can be taken only as a relative and not as an absolute measure of nicotinamide. Three values checking to  $\pm 10$  per cent are

<sup>1</sup> Type F, American Instrument Company; red filter, No. 65.

obtained before a value is assigned to an unknown material and these values are obtained on different amounts of the unknown material.

*Effect of Various Substances on the Assay*—Thiamine hydrochloride in a concentration of  $10^{-7}$  M or greater enhanced the turbidity produced by bacterial multiplication in the first 24 hours. We, therefore, incorporated thiamine hydrochloride into the basal medium. Within 48 hours, however, tubes containing no thiamine showed as good growth as those containing thiamine. In the absence of thiamine, sulfhydryl compounds (thioglycolic acid, dithioglycolic acid, and cysteine) accelerated growth in the first 24 hours. In the presence of thiamine no effect was seen with these compounds. NaCl, KCl,  $(\text{NH}_4)_2\text{SO}_4$ , and urea in concentrations up to 8 mg. per ml. of medium and  $\text{MgSO}_4$  and  $\text{MnSO}_4$  in concentrations up to 0.8 mg. per ml. of medium had neither stimulating nor depressing effects on bacterial multiplication. Pyridoxine (vitamine  $\text{B}_6$ ), riboflavin, calcium pantothenate, and vitamin  $\text{H}^2$  in concentrations up to 1  $\gamma$  per ml. of medium produced no growth in 72 hours in the absence of nicotinamide and had no effect in its presence.

*Nicotinic Acid Standards*—Although nicotinic acid will replace nicotinamide as a growth factor for dysentery bacilli, the response of our strain to increasing concentrations of nicotinic acid was slower, more irregular, and began at higher concentrations than that obtained with nicotinamide. We, therefore, could not obtain reliable standard curves with nicotinic acid under our conditions.

*Preparation of Urine for Assay*—Urine is collected without preservative for any convenient length of time. We have customarily collected 24 hour samples of urine. The volume of urine is measured. An aliquot is adjusted to approximately pH 7.0 with acetic acid or sodium hydroxide, with brom-thymol blue or litmus as outside indicators. The aliquot is transferred to a 4 ounce bottle and heated in a boiling water bath for 30 minutes, stoppered while hot with a sterile rubber stopper, and placed in the ice box until assayed. No loss of "equivalents to nicotinamide" occurred in urine stored under these conditions for 30 days unless gross bacterial contamination ensued. On the day

<sup>2</sup> A sample of "vitamin  $\text{H}$ " was obtained through the courtesy of Dr. Vincent du Vigneaud of Cornell University.

or the morning before the urine is to be assayed a known quantity of urine is transferred to a test-tube and autoclaved for 15 minutes at 15 pounds pressure. After cooling, the volume lost during autoclaving is restored by the addition of distilled water. A portion of the urine is then diluted to a convenient volume with distilled water. Dilutions of 1:20 or 1:50 are suitable for most normal urines. Highly concentrated urines sometimes require dilutions as high as 1:100.

*Results on Urine*—Lataste (7) in 1939 found that heating urine or allowing it to stand at room temperature for several days increased the nicotinamide values found by the *Proteus* assay. She ascribed the increase to hydrolysis of compounds of nicotinamide or nicotinic acid. Our experiments on both dog and human urines

TABLE I  
*Effect of Heating on Nicotinamide Equivalent Value of Urine*

Specimen No.	Before heating	Autoclaved 15 min. at 15 lbs. pressure	Autoclaved 30 min. at 15 lbs. pressure	Autoclaved 1 hr. at 15 lbs. pressure
	<i>γ per ml.</i>	<i>γ per ml.</i>	<i>γ per ml.</i>	<i>γ per ml.</i>
1	1.50	2.9	2.9	2.85
2	0.6	1.8	1.7	1.8
3	1.35	2.35	2.2	2.30
4	0.42	1.24	1.20	1.31
5	0.55	0.9	0.88	0.89

also showed an increase in the "nicotinamide equivalent" values after heating. Autoclaving the urine for 15 minutes at 15 pounds produced a maximal increase in the values found. Variations in the hydrogen ion concentrations between pH 5.0 and 8.0 had no effect on the increase in "nicotinamide equivalents" produced by autoclaving. Below pH 5.0 or above pH 8.0 the "nicotinamide equivalent" values were lower than those found on unautoclaved urine. We have, therefore, adjusted all urines to pH 7.0 and autoclaved them prior to assay. Table I presents the results obtained on five urines after autoclaving.

Assays on the 24 hour urines of eleven normal men showed outputs ranging from 1035 to 4000 "microgram equivalents to nicotinamide" per day. Oral ingestion of 30 to 50 mg. of nicotinamide by the same men produced no noticeable increase in the



24 hour excretion. The results obtained on five normal men are shown in Table II.

Assays were carried out for 6 consecutive days on the 24 hour urines of nine apparently healthy female inmates of a mental institution, who were eating a good diet. The results varied

TABLE II

*Daily Urinary Output of Nicotinamide Equivalents by Normal Men Eating Their Usual Diets*

Subject No.	Nicotinamide equivalents per 24 hrs.		
	Diet only*	Diet + 30 mg. nicotinamide	Diet + 50 mg. nicotinamide
	$\gamma$	$\gamma$	$\gamma$
1	2458 (1975-3788)	2470	3688
2	1420 (1035-1880)	1592	
3	1805 (1352-2280)	1230	3128
4	1610 (1200-2180)		1241
5	2460 (2250-2680)		2366

\* The figures not in parentheses represent the average of 2 or more days; the figures in parentheses represent the range found.

TABLE III

*Daily Urinary Excretion of "Equivalents to Nicotinamide" by Women Eating Institution Diet*

Subject No.	Average output per day	Range
	$\gamma$	$\gamma$
91	2196	1350-3020
92	1713	1090-3150
93	1776	1465-2090
94	2783	1700-3600
95	2360	2030-2600
96	3530	2510-4490
98	2435	1393-2900
99	3147	2910-3800
100	2946	2360-4970

between 1090 and 4970 "microgram equivalents to nicotinamide" per day (Table III).

Twelve normal dogs ranging in body weight from 5 to 14 kilos and eating a stock diet excreted 335 to 1095 "microgram equivalents to nicotinamide" per day. After the oral ingestion of 100 mg.

of nicotinamide, these same dogs excreted 9700 to 17,000 "microgram equivalents to nicotinamide" per day.

Assays on both dog and human urines to which nicotinamide had been added showed that the added nicotinamide could be recovered

TABLE IV  
*Recovery of Nicotinamide Added to Urine*

Subject No.	Value per ml. urine	Nicotinamide added	Diluted to	Value expected per ml. dilution	Value found per ml. dilution	Per cent recovery
	$\gamma$	$\gamma$	ml.	$\gamma$	$\gamma$	
1	0.325	0.25	10	0.0575	0.058	102
1	0.325	0.5	20	0.0575	0.057	99
2	0.72	1.0	20	0.086	0.084	99
3	1.32	1.0	20	0.12	0.116	99
4	0.96	0.5	10	0.146	0.145	99
5	0.64	2.0	20	0.132	0.138	102
6	1.03	4.0	40	0.126	0.125	99
7	1.03	0.5	10	0.153	0.164	107
8	1.24	10.0	100	0.112	0.115	102
9	0.68	1.25	25	0.077	0.078	101

1 ml. of urine was taken for analysis except for the second sample of Subject 1, which was 2 ml.

TABLE V  
*Reproduction of Values Found on Urine*

Subject No.	Date of urine collection	First assay		Second assay	
		Date	Value per ml.	Date	Value per ml.
	1940	1940	$\gamma$	1940	$\gamma$
1	Feb. 12	Feb. 13	1.16	Feb. 27	1.16
2	" 12	" 13	2.00	" 27	2.00
94	July 24	Aug. 1	0.68	Aug. 4	0.7
96	" 23	" 1	2.86	" 30	2.80
99	" 25	" 4	3.16	" 25	3.04

within  $\pm 10$  per cent. The results of ten typical recovery experiments on urine are shown in Table IV.

Assays performed on the same urine at intervals varying from 3 to 30 days showed that the values obtained on a given urine were reproducible to  $\pm 10$  per cent and that no loss occurred under our conditions of storage. Results on five urines are presented in Table V.

*Preparation of Blood for Assay*—1 ml. of oxalated blood (control experiments showed that oxalate in concentrations up to 0.04 mg. per ml. of medium had no effect on the growth of the bacteria) is transferred with an Ostwald-Folin blood pipette into a 125 ml. Erlenmeyer flask and laked with 10 to 15 ml. of distilled water. 5 ml. of 0.1 N HCl are added and the mixture autoclaved for 15 minutes at 15 pounds pressure. 4.6 to 4.8 ml. of 0.1 N NaOH are added to the mixture while it is still hot from autoclaving, bringing the pH to 6.0 to 6.5 and precipitating the blood proteins. The contents of the flask are transferred after they have cooled to a 200 ml. volumetric flask or cylinder, diluted to volume with distilled water, mixed well, and filtered through a fine quantitative filter paper. The filtrate should be crystal-clear, though certain filtrates may show a faint tinge of amber color. Milky or dark brown filtrates should not be used. Reextraction of the precipitate from blood and direct assays on the precipitates showed that none of the active material in blood was precipitated with the blood proteins. After autoclaving, the flask may, if desired, be stoppered with a sterile rubber stopper and preserved in the ice box for future assay. If the mixture has been stored in the ice box, it is reheated before the proteins are precipitated. Control experiments showed no loss of "equivalents to nicotinamide" or of added nicotinamide under such conditions within 2 weeks time.

*Results on Blood*—Assays on blood to which known amounts of nicotinamide had been added showed that the added nicotinamide could be recovered within  $\pm 10$  per cent. The results of the ten typical recovery experiments are presented in Table VI.

Assays on the same bloods at varying intervals of time showed that the values found were reproducible to within  $\pm 10$  per cent. The results of five experiments are shown in Table VII.

Assays on the bloods of eleven healthy fasting dogs of both sexes that had been maintained on a stock diet gave results varying between 649 and 1240 "microgram equivalents to nicotinamide" per 100 ml. of whole blood.

Assays on the fasting bloods of 66 apparently healthy female inmates of a mental institution who were eating a good diet gave results varying between 570 and 930 "microgram equivalents to nicotinamide" per 100 ml. of whole blood. The mean value for these 66 women was 748.5 "microgram equivalents" and the standard deviation  $\pm 76.6$  "microgram equivalents."

*Procedure for Serum or Plasma*—2 to 5 ml. of perfectly clear serum or oxalated plasma are transferred to a 100 ml. Erlenmeyer flask, 5 ml. of distilled water and 5 ml. of 0.1 N HCl are added, and the mixture autoclaved for 15 minutes at 15 pounds pressure. 4.0 to 4.2 ml. of 0.1 N NaOH are added to the mixture. Following the addition of the NaOH, the mixture is heated just to boiling

TABLE VI  
*Recovery of Nicotinamide Added to 1 Ml. of Blood*

Subject No.	Value per ml. blood	Nicotinamide added	Diluted to	Value expected per ml. dilution	Value found per ml. dilution	Per cent recovery
	$\gamma$	$\gamma$	ml.	$\gamma$	$\gamma$	
454	6.32	4.0	250	0.0413	0.041	100
448	10.2	10.0	500	0.0404	0.0396	98
445	10.63	20.0	500	0.0616	0.066	107
448	11.01	10.0	500	0.042	0.044	105
445	11.80	20.0	500	0.0636	0.069	108
446	9.81	10.0	500	0.0396	0.040	102
435	5.80	4.0	200	0.049	0.053	108
454	9.07	10.0	500	0.038	0.037	97
432	7.5	5.0	250	0.05	0.048	96
435	16.02	4.0	200	0.1	0.103	103

TABLE VII  
*Reproducibility of Values Found on Blood*

Subject No.	Blood drawn	First assay		Second assay	
		Date	Value per 100 ml.	Date	Value per 100 ml.
	1940	1940	$\gamma$	1940	$\gamma$
435	May 14	May 15	629	May 21	585
454	" 21	" 22	620	June 4	632
462	July 22	July 23	720	July 30	680
466	Aug. 12	Aug. 13	720	Aug. 27	660
454	" 12	" 20	885	" 26	906

to coagulate the serum proteins further. After cooling, the contents of the flask are transferred to a volumetric cylinder or flask and sufficient distilled water added to make a final dilution of 1:10. The contents of the flask are mixed well and filtered through a fine quantitative filter paper.

*Results on Serum*—Assays on sera to which known amounts of

nicotinamide were added showed that the added nicotinamide could be recovered within  $\pm 10$  per cent. Assays on the sera of eleven normal fasting dogs that were being maintained on a stock diet gave results varying between 12 and 35 "microgram equivalents to nicotinamide" per 100 ml. of serum.

*Preparation of Spinal Fluid for Assay*—Spinal fluid of normal protein content is simply diluted 1:10 and assayed directly. In the presence of abnormal amounts of protein, the procedure detailed for the preparation of serum is followed.

*Results on Spinal Fluid*—Assay on the spinal fluids of six healthy dogs gave results varying between 15 and 27 "microgram equivalents to nicotinamide" per 100 ml. of spinal fluid. The spinal fluids of ten women inmates of a mental institution gave results varying between 8 and 12 "microgram equivalents" per 100 ml. of spinal fluid.

#### DISCUSSION

The method which has been described is open to two criticisms: first, the necessity of expressing results in "equivalents to nicotinamide" makes quantitative comparison with the results obtained by chemical methods impossible and, second, there is no absolute proof that the test organism may not respond to some substance totally unrelated to nicotinamide. There is evidence, however, that the results obtained by bacterial methods can serve as relative measures of the level of nicotinamide and related compounds. Koser and his collaborators (10-12) examined the activity of a large number of compounds and found that dysentery organisms respond, with two possible exceptions, only to those compounds of nicotinic acid or nicotinamide which have been shown to be active in the cure or prevention of canine blacktongue or human pellagra. Only quinolinic acid and thiazole-5-carboxylic acid (13) were exceptions. The quinolinic acid was possibly contaminated with nicotinic acid owing to partial decarboxylation on heating (12). Thiazole-5-carboxylic acid was 100,000 times less active than nicotinamide (13). Snell and his coworkers (14) stress the importance of the agreement of the values obtained with different amounts of assay material as important evidence of the specificity of a bacterial assay and in this respect the method described is excellent. The good recoveries of nicotinamide added to urine,

blood, serum, and spinal fluid and the lack of non-specific stimulation or inhibition by any of the substances tested are further evidence of the reliability of the method.

The bacterial methods are superior to any of the chemical methods now in use in sensitivity, simplicity of preparation of extracts, and, above all, in the amount of work which can be performed with them. One analyst, in our experience, can carry out assays on as many as forty specimens at one time and as many as 120 assays in 1 week's time.

The range found for the 24 hour excretion of normal persons is in fair agreement with that reported by Lataste (7) who found excretions of 3 to 5 mg. of nicotinamide per day. Certain of our figures, however, are lower than any of those reported by Lataste. The difference appears to be due to individual variations in the 24 hour excretions.

Lwoff and Querido (15) using the *Proteus* test found that the blood of normal people varied from 620 to 890  $\gamma$  of nicotinamide per 100 ml. of blood. Dorfman, Koser, *et al.* (9) found that the values on five human bloods varied between 750 and 940  $\gamma$  per 100 ml. of blood. Our finding that the blood "nicotinamide equivalent" level of 66 women ranged from 590 to 930  $\gamma$  per 100 ml. of blood is in good agreement with the reports of both of these groups of investigators.

#### SUMMARY

1. A quantitative assay method for nicotinamide and related compounds in blood, urine, serum or plasma, and spinal fluid is described.

2. The method was shown to be sensitive to several hundredths of a microgram of nicotinamide and related compounds. The values obtained are reproducible to within  $\pm 10$  per cent. Recoveries of nicotinamide added to the test materials could be made within  $\pm 10$  per cent.

3. Eleven normal men excreted 1800 to 4000 "microgram equivalents to nicotinamide" per day. Nine apparently healthy female inmates of a mental institution excreted 1090 to 4970 "microgram equivalents" per day.

4. Twelve normal dogs eating a stock diet excreted 335 to 1095 "microgram equivalents" per day.

5. The blood level of 66 apparently healthy female inmates of a mental institution varied between 590 and 930 "microgram equivalents" per 100 ml. of blood.

6. The blood level of eleven normal dogs eating a stock diet varied between 649 and 1240 "microgram equivalents" per 100 ml. of blood.

7. The sera of six normal dogs varied between 12 and 35 "microgram equivalents" per 100 ml. of serum.

8. The spinal fluid of ten women inmates of a mental institution varied between 8 and 12 "microgram equivalents" per 100 ml. of spinal fluid. The spinal fluid of six healthy dogs varied between 12 and 35 "microgram equivalents" per 100 ml.

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# CHEMOIMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

## V. THE STRUCTURE OF THE TYPE III POLYSACCHARIDE

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(Received for publication, February 19, 1941)

It is the capsular polysaccharide which endows the pneumococcus with type specificity and it is this same substance which envelops the bacterial cell and renders it difficult of destruction by the phagocytes of the invaded host. When rabbits are injected with suspensions of these heat-killed, intact microorganisms, the various antibodies engendered are directed toward a number of the constituents of the bacterium. But it is the antibody elicited by the capsular polysaccharide antigen which confers type-specific immunity on experimental animals against infection with virulent pneumococci. Presumably the antibody combines chemically with the capsular polysaccharide *in situ* and renders the microorganisms susceptible to destruction by the natural protective mechanisms of the host (1).

This remarkable property of specificity exhibited by the many pneumococcal types is conditioned solely by the chemical constitution of the individual carbohydrate molecules which constitute the encapsulating material. It is, therefore, only through a knowledge of their intricate chemical make-up that one can eventually gain an understanding of the factors which govern the specificity of the bacterial polysaccharides and of the immunobiological properties of the microorganisms from which they are derived.

The capsular polysaccharide of Type III pneumococcus can be obtained from bacterial cultures in a high state of purity (2). The substance is a polybasic acid, non-diffusible, and is constituted from the elements carbon, hydrogen, and oxygen. Considerable progress in the elucidation of the structure of the polysaccharide



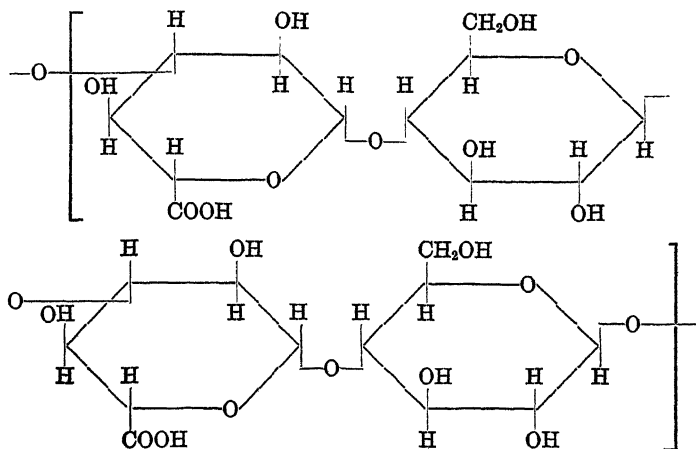
was made when it was shown to be built up from units of an aldobionic acid, 4- $\beta$ -glucuronosidoglucose or cellobiuronic acid (3). In the intact carbohydrate the cellobiuronic acid units are linked through the reducing group of the aldobionic acid to one of the hydroxyl groups of a similar unit. The objective of the present investigation is to determine the position of the intermolecular linkage of the cellobiuronic acid units.

The procedure of experimentation is that which is well established and so successfully used in studying the structure of carbohydrates in general; namely, methylation of the free hydroxyl groups of the polysaccharide followed by acid hydrolysis and identification of the partially methylated sugars found in the hydrolysate. In the present investigation we have found it advisable first to reduce catalytically the fully methylated polysaccharide. The carbomethoxyl groups of the methylated polysaccharide are thus converted to primary carbinol groups. On hydrolysis of this methylated reduced polysaccharide the uronic acid constituent appears in the hydrolysate as a dimethylglucose, whereas the glucose constituent of the aldobionic acid unit of the parent polysaccharide is isolated as 2,3,6-trimethylglucose.

The specific polysaccharide of Type III pneumococcus is readily methylated with dimethyl sulfate and sodium hydroxide and the carboxyl groups esterified with diazomethane. In this way a neutral and completely methylated polysaccharide is obtained. This substance is now reduced at high temperature and pressure, with barium-copper chromite as catalyst. Hydrolysis of this product yields equimolecular amounts of di- and trimethylglucose. These substances are converted to the corresponding methylglucosides and separated by the usual procedures. Hydrolysis of the trimethylmethylglucosides yields the known crystalline 2,3,6-trimethylglucose. From the dimethylmethylglucoside portion two crystalline products have been obtained. One of these is identical with an authentic sample of a synthetic 2,4-dimethyl- $\beta$ -methylglucoside (4); the second crystalline product has been identified as the isomeric  $\alpha$  form.

With the identification of 2,4-dimethyl- $\alpha$ - and  $\beta$ -methylglucosides as products of hydrolysis of the reduced methylated polysaccharide it becomes apparent that the methylated aldobionic acid units are linked through position 3 of the methylated glu-

curoic acid. Consideration of the data now at hand supports a structure of the Type III pneumococcus polysaccharide wherein glucose is linked to the 3rd carbon atom of the glucuronic acid while the latter in turn is known to be linked to the 4th carbon atom of a second glucose molecule (3).



In the accompanying graphic formula the glucuronosidic linkage has been definitely established as having the  $\beta$  configuration. The configuration of the linkage between the aldobionic acid units (*i.e.*, the glucosidic linkage) is assumed to be of the same type. The justification for this resides in the fact that the specific polysaccharide itself is levorotatory and on acid hydrolysis, under conditions in which the glucosidic, but not the glucuronosidic linkages, are attacked, the rotation of the solution changes from *levo* to *dextro* (2).

It is not unusual to find naturally occurring polysaccharides in which the glycosidic linkage is in position 3 (5). Most notable of those in which glucose is linked in position 3 is the polysaccharide isolated by Zechmeister and Toth from yeast cells (6). In the case of the Type III pneumococcus carbohydrate it is of interest to find the linkages of the saccharide units alternating between positions 3 and 4.

With the elucidation of this problem we have established the chemical constitution of the first of the capsular polysaccharides of an important group of pathogens. It is hoped that further work

in this field will eventually give us a more thorough understanding of the chemical basis underlying the specificity of the many types of pneumococci.

#### EXPERIMENTAL

*Methylation of Type III Polysaccharide*—10.0 gm. of the polysaccharide were dissolved in 100 cc. of 0.3 N sodium hydroxide and methylated with dimethyl sulfate and sodium hydroxide in the usual manner (7). The solution was neutralized with dilute sulfuric acid, cooled to 50°, acidified with 4 cc. of 10 N sulfuric acid, and the heavy precipitate extracted with warm butanol. The butanol extract was washed free from sulfate ions and the methylated polysaccharide recovered by evaporating the solvent *in vacuo*. The residual methylated carbohydrate was dissolved in acetone and precipitated when poured into cold petroleum ether. 12.1 gm. of material were recovered. The product had a methoxyl content of 33.85 per cent and contained some sodium ions. 10.9 gm. of this material were remethylated, yielding 9.7 gm. of a product having a methoxyl content of 36.55 per cent. This material was further methylated with methyl iodide and silver oxide (8). 8.2 gm. of the methyl ester of the methylated polysaccharide were recovered. Because this material was still low in methoxyl content, the product was acetylated with pyridine and acetic anhydride. The product obtained from this reaction was dissolved in acetone and treated with dimethyl sulfate and sodium hydroxide in the usual manner. 7.6 gm. of the methylated polysaccharide were finally obtained.

*Analysis*— $[C_{11}H_{12}O_4(OCH_3)_6COOH]_n$

Calculated.  $CH_3O$  38.0, acid equivalent 408

Found. " 37.5, " " 413

*Rotation*— $[\alpha]_D^{25} = -35.8^\circ$  ( $c = 2.0\%$  in  $CHCl_3$ -absolute alcohol 4:1)

*Methyl Ester of Methylated Polysaccharide*—7.6 gm. of the above product were dissolved in 400 cc. of methanol. An ethereal solution of diazomethane was added in slight excess. The solvents were removed and 7.96 gm. of an amorphous product melting at 185–200° were recovered. The methyl ester of the methylated polysaccharide is soluble in the usual organic solvents with the

exception of petroleum ether. The material is only slightly soluble in water.

*Analysis*— $[C_{18}H_{30}O_{11}]_n$

Calculated,  $CH_3O$  44.08; found,  $CH_3O$  43.35

*Saponification with Dilute Alkali*—Calculated. Equivalent weight 422

Found. " " 423

*Rotation*— $[\alpha]_D^{25} = -36.8^\circ$  in  $CHCl_3$  ( $c = 1.0\%$ )

*Catalytic Reduction of Methylated Polysaccharide*—1.06 gm. of the methylated polysaccharide methyl ester were dissolved in 75 cc. of methanol and 1 gm. of barium-copper chromite catalyst (9) was added. Reduction of the polysaccharide was carried out with hydrogen at  $175^\circ$  under a pressure of 3200 pounds per sq. inch. Complete reduction was effected in 20 hours. After completion of the reaction the catalyst was removed by filtration and the solution treated with norit, filtered, and evaporated to dryness. The residue weighing 800 mg. was a brittle, colorless solid insoluble in ether and petroleum ether, but soluble in chloroform, alcohol, and cold water. The substance reduced Fehling's solution only after acid hydrolysis, and the hydrolysate failed to give the naphthoresorcinol test for uronic acid. A sample of the reduced material failed to react with dilute alkali, indicating the absence of ester groups.

$[C_{17}H_{30}O_{10}]_n$ . Calculated,  $CH_3O$  39.3; found,  $CH_3O$  37.9

*Rotation*— $[\alpha]_D^{25} = -31.0^\circ$  in  $H_2O$  ( $c = 0.7\%$ )

$[\alpha]_D^{25} = -15.6^\circ$  "  $CHCl_3$  (" = 0.6%)

*Hydrolysis of Reduced Methylated Polysaccharide*—290 mg. of the reduced polysaccharide were dissolved in 2 cc. of cold concentrated hydrochloric acid and allowed to stand at room temperature overnight. The solution was then diluted to 10 cc. with water and boiled under a reflux for 6 hours. After decolorization with norit and filtration, the colorless solution was neutralized with barium carbonate and concentrated to dryness *in vacuo*. Extraction of the dry salts with acetone yielded 298 mg. of a colorless syrup. The latter, which contained the partially methylated hydrolytic products, was heated in a sealed tube at  $70-75^\circ$  with methanol containing 1 per cent dry hydrogen chloride. The solution was neutralized with solid barium carbonate, and

most of the barium chloride which formed was precipitated by the addition of a large amount of acetone. The solution was evaporated to dryness and the residue dissolved in water. The products of reaction were partitioned between chloroform and water, the trimethylmethylglucosides dissolving in the chloroform phase and the dimethylmethylglucosides remaining in the aqueous phase.

After hydrolysis of the product from the chloroform extract a 50 per cent yield of crystalline 2,3,6-trimethylglucose was obtained. The derivative melted at 113° and the melting point was not depressed when the substance was mixed with an authentic sample.

$C_9H_{18}O_6$ . Calculated,  $CH_3O$  41.9; found,  $CH_3O$  40.8  
*Rotation*— $[\alpha]_D^{25} = +89.70^\circ$ , equilibrium in  $H_2O$  ( $c = 1.3\%$ )

The aqueous phase was evaporated to dryness, yielding 117 mg. of crude dimethylmethylglucosides. When this material was dissolved in 2 cc. of ether and allowed to stand in the ice chest, 69 mg. of crystalline material separated and two different crystalline forms were obviously present. Repeated recrystallization from ether gave 23 mg. of pure 2,4-dimethyl- $\beta$ -methylglucoside, melting at 122–123°. Upon cooling the substance crystallized and remelted at 105–107°. These melting point values were not depressed when the above product was mixed with synthetic 2,4-dimethyl- $\beta$ -methylglucoside. On two other occasions a similar yield of this substance was obtained upon hydrolysis of the reduced methylated polysaccharide.

$C_9H_{18}O_6$ . Calculated,  $CH_3O$  41.9; found,  $CH_3O$  41.2  
*Rotation*— $[\alpha]_D^{20} = -16.5^\circ$  in acetone ( $c = 0.5\%$ )

After separation of as much 2,4-dimethyl- $\beta$ -methylglucoside as possible the mother liquors on long standing in the ice box deposited 15 mg. of crystals in the form of clusters. This substance melted at 79–81°. When mixed with 2,3-dimethyl- $\alpha$ -methylglucoside (10) (m.p. 83–84°), the mixture melted at 65–75°, but when mixed with an authentic sample of 2,4-dimethyl- $\alpha$ -methylglucoside, the melting point was not depressed.

$C_9H_{18}O_6$ . Calculated. C 48.65, H 8.11,  $CH_3O$  41.89  
 Found. " 48.45, " 7.92, " 41.66  
*Rotation*— $[\alpha]_D^{20} = +159^\circ$  in acetone ( $c = 0.3\%$ )

The sample taken for optical rotation measurement was observed to be contaminated with a small amount of the crystals of the  $\beta$ -methylglucoside.

*2,4-Dimethyl- $\alpha$ -Methylglucoside*—By methylation of 6-trityl- $\alpha$ -methylglucoside and subsequent detritylation Robertson and Waters obtained, in addition to 2,3,4-trimethyl- $\alpha$ -methylglucoside, a substance of unknown structure which melted at 79° (11). A sample of the latter material was therefore prepared. On purification the material was found to be identical with the high rotating dimethylmethylglucoside obtained by us from the hydrolytic products of the reduced methylated Type III polysaccharide. The substance melted at 79–80° and the melting point was depressed when mixed with known 2,3-dimethyl- $\alpha$ -methylglucoside.

$C_9H_{18}O_6$ . Calculated. C 48.65, H 8.11,  $CH_3O$  41.89  
Found. " 48.89, " 8.16, " 41.73

Rotation— $[\alpha]_D^{25} = +186^\circ$  in acetone ( $c = 1.0\%$ )

*Conversion of 2,4-Dimethyl- $\alpha$ -Methylglucoside to  $\beta$  Form*—A solution of 110 mg. of the new dimethyl- $\alpha$ -methylglucoside in 2 cc. of methanol containing 2.5 per cent HCl was heated in a sealed tube at 100° for 16 hours. The solvent was removed *in vacuo* and the residue twice evaporated after the addition of small amounts of toluene. When this product was dissolved in ether, the solution yielded 15.9 mg. of crystals melting at 121.5–123° (corrected). The product was identical with the high melting dimethylmethylglucoside obtained from the hydrolytic products of the methylated Type III polysaccharide and was also identical with the dimethyl- $\beta$ -methylglucoside recently synthesized by an entirely different procedure (4). All of these substances therefore have their fixed methyl groups in the same position. By the following experiments they are shown to be derived from 2,4-dimethylglucose.

*Proof of Structure of 2,4-Dimethyl- $\alpha$ - and  $\beta$ -Methylglucosides*—It has been shown by Brigl and Schinle (12) that osazones can be formed from aldoses methylated in position 2. This reaction involves the removal of the methoxyl group and is regarded as evidence that the methoxyl was originally substituted in position 2. In order to establish the structure of the 2,4-dimethylglucose derivatives isolated in this study, the latter were converted to

the known 4-methylglucosazone. In this manner final proof of the structure of these derivatives was established.

The 94.1 mg. of mixed glucosides remaining after isolation of the crystalline  $\beta$ -methylglucoside in the previous experiment were hydrolyzed in a sealed tube at  $100^\circ$  for 16 hours. 320 mg. of crystalline sodium acetate and 184 mg. of phenylhydrazine hydrochloride were now added. The solution, after removal of a small precipitate, was heated on a water bath for 2.5 hours. The oil which separated was dissolved in dilute alcohol. The crystals of osazone which separated on cooling were recrystallized from benzene. This osazone melted at  $156\text{--}157^\circ$  (corrected) and the melting point was not depressed when mixed with authentic 4-methylglucosazone.

$[C_{18}H_{21}O_8N_4(OCH_3)]$ .	Calculated.	$CH_3O$	8.34
	Found.	"	7.95

*4-Methylglucosazone*—Crystalline 4-methyl-2,3,6-triacetyl- $\beta$ -methylglucoside (13) was hydrolyzed and treated with sodium acetate and phenylhydrazine hydrochloride under the conditions described above. The osazone crystallized from dilute alcohol and was recrystallized from benzene. It melted at  $156\text{--}157^\circ$  (corrected). Munro and Percival (14) and Schinle (15) have observed  $158^\circ$  and  $159^\circ$ , respectively, as the melting point of 4-methylglucosazone.

#### SUMMARY

1. The methylation of the capsular polysaccharide of Type III pneumococcus and its catalytic reduction have been described.

2. Hydrolysis of the reduced polysaccharide yields the known 2,3,6-trimethylglucose and two substances identified as 2,4-dimethyl- $\alpha$ - and  $\beta$ -methylglucosides. Both of these substances have been synthesized and their structures confirmed by an independent series of reactions.

3. From the hydrolysis products of the reduced methylated polysaccharide it has been possible to establish the position of linkage between the aldobionic acid units in the intact polysaccharide.

The catalytic reductions were carried out with the generous cooperation of the late Dr. P. A. Levene to whom the authors are also

indebted for the sample of 4-methyl-2,3,6-triacetyl- $\beta$ -methylglucoside.

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# CHROMATOGRAPHIC SEPARATION AND COLORIMETRIC DETERMINATION OF ALCOHOLIC AND NON-ALCOHOLIC 17-KETOSTEROIDS IN EXTRACTS OF HUMAN URINE\*

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Many isolation experiments have recently been reported in which the 17-ketosteroids in extracts of large batches of human urine have been separated and identified in crystalline form.<sup>1</sup> The evidence obtained by these investigations showed that there were significant quantitative and qualitative differences between the steroid content of urine from normal and pathological individuals. Although such detailed separations appear to give important information, their application to single 24 hour specimens of urine is impractical. For this reason attempts were made to devise simple rapid procedures for the detailed analyses of urinary ketosteroids which could be applied to 24 hour urine extracts.

In previous communications a colorimetric method for assaying the total,  $\alpha$ -, and  $\beta$ -<sup>2</sup> neutral urinary 17-ketosteroids was described (2) and evidence was presented which showed that the determina-

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† Research Fellow on a grant from the National Cancer Institute administered by Professor L. F. Fieser.

<sup>1</sup> For a summary of these reports see Wolfe, Fieser, and Friedgood (1).

<sup>2</sup> In those papers the term  $\beta$  fraction referred to the 3- $\beta$ -hydroxyketosteroids which were precipitated with digitonin. For brevity and convenience the term  $\alpha$  fraction was used to refer to the remaining ketosteroids which were not precipitated with digitonin. Since this method was described (2), it has been found that the non-alcoholic 3-chlorodehydroisoandrosterone appears quantitatively in the  $\beta$  fraction.

tion of their relative concentrations in human urine was of diagnostic value (3).

A well known procedure for separating alcoholic and non-alcoholic steroids involves esterification of the alcohols with succinic or phthalic anhydride in the presence of anhydrous pyridine to form the half esters and their subsequent removal from the mixture as sodium salts with carbonate solution (4, 5). Non-alcoholic substances are unchanged by such a process and remain in the organic solvent. The alcohols may be recovered from the esters by saponification with alcoholic potassium hydroxide. We have carried out several such separations on extracts from 24 hour urine specimens, but found the procedure to be impractical for routine clinical analysis. An alternative procedure was suggested by the fact that alcoholic ketosteroids are adsorbed much more firmly upon a column of activated alumina from an inert organic solvent than are non-alcoholic ketosteroids (1). Activated alumina has been used by several investigators in the separation of urinary steroids (6, 7), but so far no attempt has been made to develop a convenient assay procedure based on its use.

The data presented in this paper show that reasonably accurate determinations of the relative concentrations of alcoholic and non-alcoholic ketosteroids present in 24 hour urine extracts can be made by percolating a carbon tetrachloride solution of the steroids through a standardized column of activated alumina. The procedure involves (a) the colorimetric determination of the total alcoholic and non-alcoholic ketosteroids in the original sample,<sup>3</sup> (b) selective adsorption of the alcoholic ketosteroids upon a column of activated alumina, and (c) colorimetric determination of the non-alcoholic ketosteroid content of the eluate from the column. This paper thus reports a simple procedure for determining the respective concentrations of total,  $\alpha$ - and  $\beta$ -alcoholic, and non-alcoholic urinary neutral 17-ketosteroids in a single 24 hour urine sample. Because preliminary observations on the daily excretion of total,  $\alpha$ - and  $\beta$ -alcoholic, and non-alcoholic 17-ketosteroids by normal and abnormal individuals reveal that the output of the various constituents varies independently, it is

<sup>3</sup> Equimolar amounts of alcoholic or non-alcoholic 17-ketosteroids react with *m*-dinitrobenzene in the presence of absolute ethanolic KOH solution to give identical colors (8, 9).

suggested that each constituent may have a different physiologic significance.

The latest evidence to be found in the literature suggests that at least three non-alcoholic 17-ketosteroids may be present in extracts of acid-hydrolyzed human urine.  $\Delta^3,5$ -Androstadienone-17 has been isolated from the urine of patients of both sexes with carcinoma of the adrenal cortex (1, 10).  $\Delta^2$ -Androstenone-17 has been obtained from pooled urine of castrated females (7). 3-Chlorodehydroisoandrosterone has been isolated from the hydrolyzed urine of a female patient with carcinoma of the adrenal cortex (1).

Although the isolation of these substances from extracts of human urine is of great interest, the possibility has been raised that they are not body metabolites, but that they may have been formed from other 17-ketosteroids such as androsterone or dehydroisoandrosterone during the period of acid hydrolysis. Hirschmann has shown that  $\Delta^2$ -androstenone-17 may be prepared from androsterone in the laboratory (7). Butenandt *et al.* have noted that hydrochloric acid hydrolysis results in some chlorination of the 3-carbon atom of  $\Delta^5$ -dehydroisoandrosterone (11). Certain types of compounds such as  $\Delta^4$ -dehydroisoandrosterone may be expected to be dehydrated easily in the presence of hydrochloric acid (as dilute as N/30) and thus be converted into non-alcoholic substances (12). The presence of an as yet unisolated substance of this type may account for the occurrence of non-alcoholic steroids in simultaneously extracted and hydrolyzed specimens.

Talbot *et al.* (13) also presented evidence which showed that brief hydrolysis of urine with hydrochloric acid affected the 3-hydroxyl group of 17-ketosteroids. These observations have been extended in the present paper, showing that acid hydrolysis converts some androsterone and dehydroisoandrosterone into substances which are no longer separable from non-alcoholic ketosteroids by adsorption on activated alumina. These destructive effects of hydrolysis are largely eliminated by the simultaneous hydrolysis and extraction procedure described previously (13). Nevertheless, non-alcoholic 17-ketosteroids have been found in extracts obtained by the simultaneous extraction and hydrolysis procedure.

## EXPERIMENTAL

*Reagents—*

Activated alumina "according to Brockmann"<sup>4</sup> (Merck, Darmstadt).

Absolute carbon tetrachloride (c.p., Baker's analyzed).

*Apparatus*—The adsorption column and pressure bottle used are shown in Fig. 1. To charge the column with alumina the apparatus is dried in a warm oven and the flask *B* is removed. A cotton or glass wool plug is introduced into the small bulb *E*. The tube is then filled with 20 to 30 mm. increments of alumina until the column is 165 to 170 mm. in height (*A*), each portion being gently tapped after addition.

The carbon tetrachloride containing the 17-ketosteroids is then placed in the reservoir *B* and an air pressure of 18 to 20 cm. of mercury is applied and maintained by means of the pressure bottle shown in the diagram.

*Procedure*

The urine was collected with 7 cc. of concentrated hydrochloric acid per liter as a preservative. Except when otherwise stated,<sup>5</sup>

<sup>4</sup> Since submitting this paper for publication it has been found that aluminum oxide anhydrous "according to Brockmann" can be obtained from Merck and Company, Inc., Rahway, New Jersey. Preliminary experiments with this brand of alumina indicate that it is more active than the Merck, Darmstadt, alumina which was used in the experiments reported here. To compensate for the increased activity of the alumina, it is necessary to employ a stronger eluent than absolute carbon tetrachloride. c. p. benzene or c. p. benzene containing approximately 5 per cent of anhydrous ethyl ether by volume appears to be satisfactory for separating alcoholic and non-alcoholic ketosteroids. Because of these observed differences between batches of alumina it is advisable to determine the correct eluent for each new batch with crystalline samples of a non-alcoholic ketosteroid (such as cholestanone or chlorodehydroisoandrosterone) and an alcoholic ketosteroid (such as dehydroisoandrosterone) before the alumina is employed for assay purposes.

<sup>5</sup> In comparative experiments the urine was independently hydrolyzed by boiling with 15 volumes per cent of hydrochloric acid for 10 minutes followed by extraction for  $\frac{3}{4}$  hour with carbon tetrachloride (13, 14).

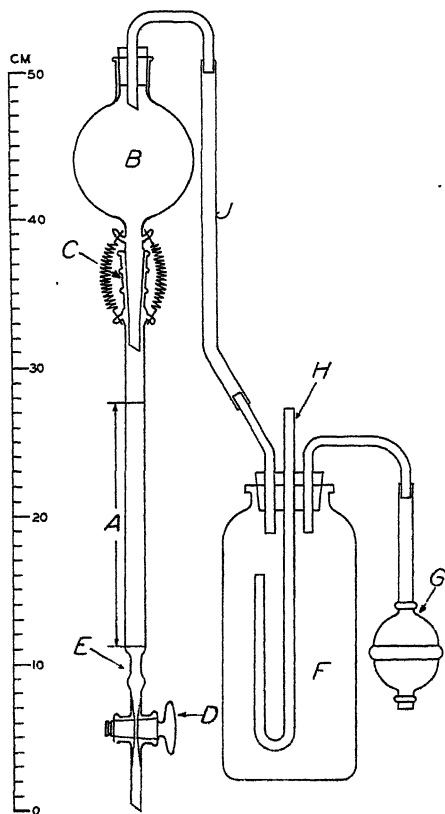


FIG. 1. Chromatographic adsorption apparatus. *A* designates the alumina portion of the tube, 10 mm. inside diameter and 165 to 170 mm. in length. Flask *B* is a glass reservoir of 250 cc. capacity fitted with a rubber stopper containing an inlet tube. *C* is a No. 14/35 ground glass joint held tightly in place by two steel springs. *D* is a stop-cock No. 12A with a 1 mm. bore in the plug, No-Lub; Scientific Glass Apparatus Company, Bloomfield, New Jersey. Bulb *E* is packed with cotton or glass wool and acts as a filter mat for the alumina. *F* is a pressure bottle made from a large laboratory reagent container and equipped with a 3-hole rubber stopper. *G* is a pressure bulb of rubber with a ball valve in the lower end. *H* is a manometer tube with the upper end open to the atmosphere and the lower one open in the pressure bottle. This tube is filled with mercury to about 10 to 11 cm. A pressure of 10 to 20 cm. of mercury is maintained on the liquid in *B* while solvent is passing through. *J* is a rubber connecting tube.

the simultaneous hydrolysis and extraction procedure described elsewhere (13) was employed. The neutral residue was then obtained and separated into ketonic and non-ketonic components with Girard's Reagent T (13). The ketonic fraction was dissolved in a measured quantity of absolute ethanol and aliquots were taken for (a) colorimetric assay of the total 17-ketosteroid content, (b) separation and colorimetric determination of the 3- $\beta$ -hydroxy-ketosteroid concentration by the digitonin precipitation procedure reported elsewhere (2), and (c) separation and colorimetric determination of the non-alcoholic constituents.

For the determination of the relative concentrations of alcoholic and non-alcoholic constituents, aliquot (c) was treated as follows: The solvent was evaporated on the water bath. Removal of last traces of the solvent was facilitated by the addition and subsequent evaporation of small portions of anhydrous ethyl ether. The residual gum was placed in a vacuum desiccator over calcium chloride for a period of several hours. The dried gum was dissolved in a measured volume of carbon tetrachloride (2 cc. or more per mg. of 17-ketosteroid) with the aid of heat if necessary. The solution was transferred quantitatively to the reservoir at the top of the column. To increase the rate of flow, pressure was applied with the pressure bottle and the solution allowed to flow until the meniscus had reached a level approximately 1 cm. above that of the alumina. Fresh solvent (150 cc.) was then introduced into the reservoir and the same procedure repeated. The entire eluate was collected in a flask at the bottom of the column (non-alcoholic fraction). A new flask was then substituted at the bottom of the column and an additional 50 cc. lot of fresh solvent was percolated through the column (control fraction).

The 17-ketosteroid content of an aliquot of the non-alcoholic fraction and the control fraction was determined colorimetrically (2, 13) after the solvent was evaporated off and the residue dissolved in a measured quantity of absolute ethanol.

If the control fraction contained any 17-ketosteroid (indicating that alcoholic ketosteroids were being eluted from the column), the non-alcoholic fraction was dried, dissolved in carbon tetrachloride, and re-percolated through a column of fresh alumina as described above. Otherwise, the fact that there were no 17-

ketosteroids in the control fraction was considered to be satisfactory evidence that no alcoholic 17-ketosteroids had been eluted from the alumina. A single column of alumina could be used repeatedly until ketosteroids appeared in the control fraction.

### Results

*Rate of Elution of Non-Alcoholic Ketosteroids*—Fig. 2 shows the approximate volume of carbon tetrachloride required to elute representative non-alcoholic ketosteroids from the column of alumina. In one experiment a solution of 24 mg. of cholestanone in 15 cc. of solvent was passed through the column. When the meniscus almost reached the top surface of the alumina, fresh

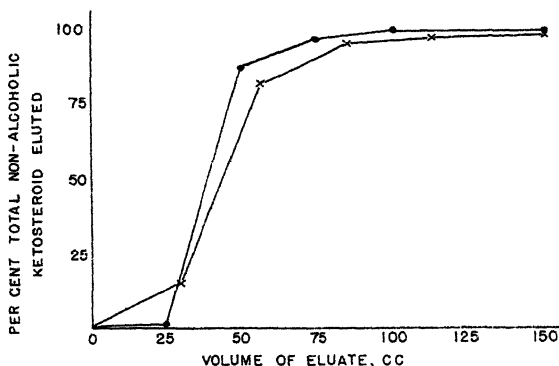


FIG. 2. Volume of carbon tetrachloride required to elute non-alcoholic ketosteroids from the alumina column.

solvent was added to the reservoir. The eluate was collected in small lots and the ketosteroid content of each lot determined colorimetrically.<sup>6</sup> The second experiment duplicated the former procedure except that 19 mg. of coprostanone were used instead of cholestanone. The results obtained indicate that these non-alcoholic ketosteroids are essentially completely eluted by 100 cc. of solvent.

*Elution of Alcoholic 17-Ketosteroid from Alumina*—A solution containing 0.1 mg. of dehydroisoandrosterone per cc. of the

<sup>6</sup> The colorimetric procedure described previously (2) was modified slightly for the determination of 3-ketosteroids. Suitable calibration curves were obtained for each 3-ketosteroid used.



carbon tetrachloride was percolated through a column, the eluate was collected in 200 cc. lots, and the 17-ketosteroid content of each fraction determined separately. Table I shows that no dehydroisoandrosterone appeared in the eluate until more than 400 cc. of the solution had been percolated through the column. Thereafter dehydroisoandrosterone appeared in gradually increasing amounts. This and other unreported data suggest that the standardized alumina column described here is capable of adsorbing at least 40 mg. of dehydroisoandrosterone, provided the concentration of the steroid in carbon tetrachloride does not exceed 0.5 mg. per cc.

TABLE I

*Adsorption and Elution of Dehydroisoandrosterone from Absolute Carbon Tetrachloride Solution Containing 0.1 Mg. of Dehydroisoandrosterone per Cc.*

Total of CCl <sub>4</sub> solution percolated through column	Total steroid placed on column	Total steroid recovered in eluate
cc.	mg.	mg.
200	20	0.0
400	40	0.0
600	60	0.04
800	80	0.09
1000	100	0.4
1200	120	3.0

*Determination of Alcoholic and Non-Alcoholic Ketosteroid Content of Known Solutions*—In these experiments the procedure described above for separating alcoholic and non-alcoholic ketosteroids was followed, with the results shown in Table II. The known ketosteroid content of the original solution is shown in Columns 1 and 2, and that appearing in the non-alcoholic fraction is given in Column 5. The amount of ketosteroid in the alcoholic fraction was determined by the difference between the ketosteroid content of the original solution and the ketosteroid content of the non-alcoholic fraction<sup>7</sup> (Column 3). The ratios of the determined to

<sup>7</sup> In Experiments 10 and 11, the alcoholic 17-ketosteroid was recovered from the alumina with absolute ethanol after the non-alcoholic ketosteroids had been eluted with carbon tetrachloride. Ethanol effectively elutes alcoholic steroids from activated alumina (1).

the theoretical values for alcoholic and non-alcoholic constituents (Columns 4 and 6) indicate that the procedure provides a reasonably satisfactory means of separating alcoholic and non-alcoholic ketosteroids.

TABLE II  
*Separation of Alcoholic and Non-Alcoholic Ketosteroids by Column of Activated Aluminum Oxide*

Experiment No.	Original ketosteroid* sample		Ketosteroid appearing after separation on alumina			
	Alcoholic (A)	Non-alcoholic (B)	Alcoholic fraction		Non-alcoholic fraction	
			Determined (C)	$\frac{C}{A}$	Determined (D)	$\frac{D}{B}$
	(1)	(2)	(3)	(4)	(5)	(6)
	mg.	mg.	mg.		mg.	
1	DHA (100)	None	100.0	1.00	0	
2	" (40)	"	40.0	1.00	0	
3	" (13)	"	13.0	1.00	0	
4	None	Cop.(19)	1.3		17.7	0.93
5	"	Chol.(24)	0.7		23.3	0.97
6	"	" (29)	0.0		30.0	1.03
7	"	Andr.(2.7)	0.2		2.5	0.93
8	"	Chlor.(15.3)	0.0		15.3	1.0
9	"	" (8.1)	0.0		8.4	1.04
10	DHA (33.5)	Chol.(47)	33.0†	0.98	46.0	0.98
11	" (33.5)	Cop.(27.7)	30.4†	0.91	26.2	0.95
12	" (34.0)	Andr.(2.2)	34.4	1.01	1.9	0.86

\* The symbols employed denote the steroid used. DHA = dehydroisoandrosterone; Cop. = coprostanone; Chol. = cholestanone; Andr. =  $\Delta^{3,5}$ -androstdienone-17; Chlor. = 3-chlorodehydroisoandrosterone. The figures in parentheses following the symbol indicate the number of mg. of ketosteroid used.

† These values were obtained by recovering the alcoholic ketosteroid from the alumina with absolute ethanol after the non-alcoholic ketosteroid had been eluted with carbon tetrachloride.

*Recovery of Alcoholic and Non-Alcoholic Ketosteroids Added to Urine Extract*—The data of Table III present determinations of total and non-alcoholic 17-ketosteroids in a pool of the ketonic fractions of extracts of normal adult urine before and after known amounts of crystalline dehydroisoandrosterone and 3-chloro-

dehydroisoandrosterone had been added. The alcoholic steroid values were calculated by difference between the total and non-alcoholic 17-ketosteroids. The control values obtained before hormone was added are given first (Experiments 1 and 2). The theoretical values represent the sum of the determined control values and the mg. of the hormone added. The corresponding determined values are listed under the control determined values. It will be seen that the theoretical and determined values agree satisfactorily. This indicates that alcoholic and non-alcoholic

TABLE III

*Recovery of Known Amounts of Crystalline Dehydroisoandrosterone and 3-Chlorodehydroisoandrosterone Added to Aliquots of Pooled Ketonic Fraction of Normal Adult Urine Extract\**

The results are expressed as mg. per unit aliquot.

Experiment No.	Ketosteroid added†	Total ketosteroids	Alcoholic ketosteroids		Non-alcoholic ketosteroids	
			Determined‡	Theoretical	Determined	Theoretical
1	None	7.0	6.5		0.5	
2	"	7.0	6.5		0.5	
3	CIDHA (15.0)	22.0	7.1	6.5	14.9	15.5
4	" (12.0)	19.0	6.8	6.5	12.2	12.5
5	DHA (7.4)	14.4	13.7	13.9	0.7	0.5
6	" (15.0)	21.8	21.4	21.3	0.4	0.5

\* Obtained by a simultaneous extraction and hydrolysis procedure.

† The following symbols denote the compound used: CIDHA = 3-chlorodehydroisoandrosterone; DHA = dehydroisoandrosterone. The figures in parentheses following the symbol indicate the number of mg. used.

‡ Total ketosteroids minus non-alcoholic ketosteroids.

17-ketosteroids may be assayed as accurately in carbon tetrachloride solutions of the ketonic fraction of urine extracts as in pure carbon tetrachloride solutions.

*Comparison of Recovery of Neutral Alcoholic 17-Ketosteroids from Urine after Various Methods of Hydrolysis and Extraction*—Because of the observations of others (11) that alcoholic ketosteroids may be converted into non-alcoholic substances by hydrochloric acid hydrolysis, the relative effects of independent hydrolysis and of simultaneous hydrolysis and extraction of urine were investigated. Recovery experiments were carried out in which known amounts

of crystalline androsterone or dehydroisoandrosterone were added to aliquots of urine, the total,  $\alpha$ - and  $\beta$ - alcoholic, and non-alcoholic content of which was known. The analytical data from a series of such recovery experiments are given in Table IV. The theoretical values represent the sum of the values of the control urine sample and the mg. of crystalline hormone added to each aliquot.

TABLE IV

*Effect of Hydrolysis Procedure upon Alcoholic 17-Ketosteroids Added to Urine Prior to Hydrolysis and Extraction*

The results are expressed as mg. per liter of urine, determined by colorimetric assays on the ketonic fraction.

Experiment No.	Ketosteroid added*	Hydrolysis and extraction†	Total 17-ketosteroids		Alcoholic 17-ketosteroids‡				Non-alcoholic 17-ketosteroids	
			Determined	Theoretical	$\alpha$ fraction		$\beta$ fraction		Determined	Theoretical
					Determined	Theoretical	Determined	Theoretical		
1A	None	I	9.8		8.6		0.3		0.9	
2A	DHA (20)	"	18.3	29.8	4.9	8.6	7.5	20.3	5.9	0.9
3A	A (7.6)	"	16.6	17.4	13.5	16.2	0.8	0.3	2.3	0.9
4A	None	S	8.7		7.9		0.0		0.8	
5A	DHA (20)	"	27.3	28.7	9.0	7.9	17.5	20.0	0.8	0.8
1B	None	"	4.5		3.2		0.7		0.6	
2B	DHA (20)	"	23.4	24.5	3.4	3.2	19.6	20.7	0.4	0.6

\* The symbols employed refer to the following substances: DHA = dehydroisoandrosterone; A = androsterone. The values in parentheses indicate the number of mg. added.

† The symbol I refers to independent hydrolysis followed by extraction; the symbol S to simultaneous extraction and hydrolysis (13).

‡ The  $\alpha$  fraction was calculated by subtracting the sum of the  $\beta$  fraction plus the non-alcoholic fraction from the total. The  $\beta$  fraction was determined by the method reported elsewhere (2).

The data show that independent hydrolysis converts a portion of the added crystalline dehydroisoandrosterone and androsterone (Experiments 2A and 3A) to non-alcoholic ketosteroids which are not adsorbed by alumina; Experiment 2 also confirms previously reported experiments (13) which indicated that independent acid hydrolysis results in some destruction of the unconjugated hormone, as evidenced by a decrease in total color-producing sub-

stances and some replacement of the 3- $\beta$ -hydroxyl group on which the estimation of the  $\beta$  fraction depends.

The data of Experiments 4A and 5A and 1B and 2B further show that the above effects of hydrolysis are largely eliminated by simultaneous extraction and hydrolysis.

TABLE V

*Excretion of Total,  $\alpha$ - and  $\beta$ - Alcoholic, and Non-Alcoholic 17-Ketosteroids by Normal and Abnormal Individuals*

The results are expressed as mg. of 17-ketosteroids per 24 hours or per liter\* as determined colorimetrically on the ketonic fraction of simultaneously extracted and hydrolyzed urine.

Specimen No.	Type of urine	17-Ketosteroids				
		Total	Alcoholic		Non-alcoholic	
			$\alpha$ -	$\beta$ -	Determined	Per cent total
1	Normal adult, pooled	7.0			0.5	7
2	" " "	9.8			0.9	9
3	" " ♂, 30 yrs.	15.1	12.1	1.7	1.3	9
4	" " ♂, 45 "	9.1	8.6	0.0	0.5	6
5	" " ♀, 33 "	5.8	4.4	1.3	0.1	2
		8.0	6.1	1.5	0.4	5
		10.6	7.0	3.1	0.5	5
6	" " ♀, 20 "	6.4	5.7	0.3	0.4	6
		9.4	8.7	0.3	0.4	4
7	Adrenal hyperplasia, ♀, 11 yrs.	23.4	17.2	4.6	1.6	7
8	Probable adrenal† hyperplasia, ♂, 4½ yrs.	20.8	17.4	2.0	1.4	7
9	Adrenal hyperplasia with adrenal insufficiency, ♂, 3 yrs.‡	10.0	9.0	1.0	0.0	0
10	Adrenal carcinoma, ♀, 13 yrs.§	132.8	39.0	64.8	29.0	22

\* The data for Specimens 1, 2, and 10 are expressed as mg. per liter; the rest are expressed as mg. per 24 hours.

† We are indebted to Dr. J. Warkany for this sample.

‡ Case reported elsewhere (15).

§ We are indebted to Dr. H. B. Friedgood for this specimen.

These findings confirm our earlier conclusion that simultaneous extraction and hydrolysis of urine is essential, especially when detailed information is desired.

*Excretion of Total,  $\alpha$ - and  $\beta$ - Alcoholic, and Non-Alcoholic 17-Ketosteroids by Normal and Abnormal Individuals—Preliminary*

observations on the estimation of the various ketonic constituents of simultaneously extracted and hydrolyzed 24 hour urine samples are presented in Table V. Non-alcoholic 17-ketosteroids comprised approximately 7 per cent of the total 17-ketosteroids excreted by normal individuals and patients with adrenal hyperplasia. The excretion of non-alcoholic constituents was low in a child with the adrenogenital syndrome associated with symptoms of adrenocortical insufficiency (15) and was high in the patient with carcinoma of the adrenal cortex. The values obtained agree reasonably well with those obtained by extensive isolation experiments on extracts of pooled urine from a castrated female (7) and of urine from patients with carcinoma of the adrenal cortex (1, 10). Although the exact physiologic and clinical significance of the rate of excretion of the various constituents of the ketonic fraction of urine extracts cannot be stated until more data are obtained, the material at hand suggests that the determination of the non-alcoholic fraction may be of added value in the differential diagnosis of adrenocortical disease.

#### SUMMARY

A procedure is described for the separation and determination of alcoholic and non-alcoholic 17-ketosteroids. The procedure involves the colorimetric (*m*-dinitrobenzene reaction) determination of the ketosteroid content of the solutions before and after adsorption of the alcoholic ketosteroids upon a column of activated aluminum oxide. By this procedure and another described previously, it is possible to determine the total,  $\alpha$ - and  $\beta$ -alcoholic, and non-alcoholic 17-ketosteroid content of a single 24 hour urine specimen.

Hydrolysis of urine with hydrochloric acid affects the 3-hydroxyl group of urinary 17-ketosteroids so that these ketosteroids are no longer separable from non-alcoholic ketosteroids by adsorption on aluminum oxide. These effects of hydrolysis are largely eliminated by the simultaneous extraction and hydrolysis procedure described previously.

Preliminary observations on the excretion of total,  $\alpha$ - and  $\beta$ -alcoholic, and non-alcoholic 17-ketosteroids by normal and abnormal individuals are reported.

The authors are indebted to Dr. L. F. Fieser and Dr. A. M. Butler who made suggestions in the preparation of this paper. Dr. E. Schwenk, of the Schering Corporation, generously supplied the crystalline androsterone and dehydroisoandrosterone used herein.

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## CARBOHYDRATE STORAGE AND MOBILIZATION WITH CHANGES IN THE BLOOD pH

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The possible influence of pH on carbohydrate metabolism has aroused considerable interest. This is in part because of the likelihood of acidosis in diabetes mellitus and in part because of the theoretical effects of the pH variation on glycogenesis and glycogenolysis as well as on the stability of glycogen and glucose. It is generally accepted that the disturbed fat metabolism in diabetes is associated with an acidosis, but the effect of a lowered pH upon the storage of carbohydrate as glycogen or upon its mobilization has not been established. Here, as in many biological relationships, it is difficult to determine which is cause and which is effect.

The earlier investigations (1-3) of this subject were limited in their scope by the technical difficulties in the determination of small pH changes and because no micro sugar method was available.

More recently relationships of the pH, CO<sub>2</sub>, and lactic acid content of the blood have been described by Anrep and Cannan (4) and by others (5, 6), and a few observations have been made by Kumamura (7) on liver glycogen, blood sugar, and pH relationships in the living animal. A fairly extensive review of the literature is given by Donnelly (8).

Donnelly discussed the question from the standpoint of the chemical stabilities of glucose and glycogen toward acid and alkali respectively. While there is no doubt that glycogen and glucose react to pH changes *in vitro*, conditions *in vivo* are com-

\* Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.



plicated by enzyme activity and the dependence of this activity upon a suitable pH. The optimal pH for the activity of the various enzymes involved may be and probably is quite different from that occurring when the pH itself is the effective catalyzing agent.

If the acid-base balance is an important factor in the determination of the glycogen and glucose stabilities *in vivo*, it should be possible to show alterations in glycogen and blood sugar under carefully controlled conditions, when an adequate supply of these two carbohydrates is available in the liver and the blood and the pH is experimentally altered within physiological limits. These conditions with respect to carbohydrate are realized toward the end of the feeding period, when the liver glycogen concentration is high and carbohydrate is still being absorbed from the intestine. The present study deals with the liver and muscle glycogen concentrations, blood sugar, and pH with experimental alterations in the acid-base balance.

### *Methods*

The basis for the selection of the subjects, their preparation for the experiment, and the entire glycogen technique have been described elsewhere.<sup>1</sup> All animals in this study were maintained on the Rockland rat diet in pellet form. The 12 hour feeding interval prior to the tissue sampling was preceded by a 48 hour inanition period. Blood sugar was determined by the second Shaffer-Hartmann-Somogyi microtechnique (9). The CO<sub>2</sub> and O<sub>2</sub> contents of the blood were determined on 2 cc. by the method of Van Slyke and Neill (10).

The technique for obtaining the blood samples and determining their pH has been outlined by Rawson and Guest (11). The blood was obtained by heart puncture from the anesthetized rat. For this purpose a specially prepared syringe and needle were used. A lateral hole was drilled with a No. 43 drill through the sleeve of a 20 gage, 1.5 inch needle, near enough to the shaft to be beyond the inserted syringe tip. To prepare the syringe to receive the blood a drop of mercury and a small amount of a saturated solution of neutral potassium oxalate were placed in the barrel. The excess oxalate with any air bubbles was forced out and the

<sup>1</sup> Guest, M. M., *J. Nutrition*, in press.

plunger brought into contact with the end of the barrel. By this means the syringe tip was filled with mercury and the needle

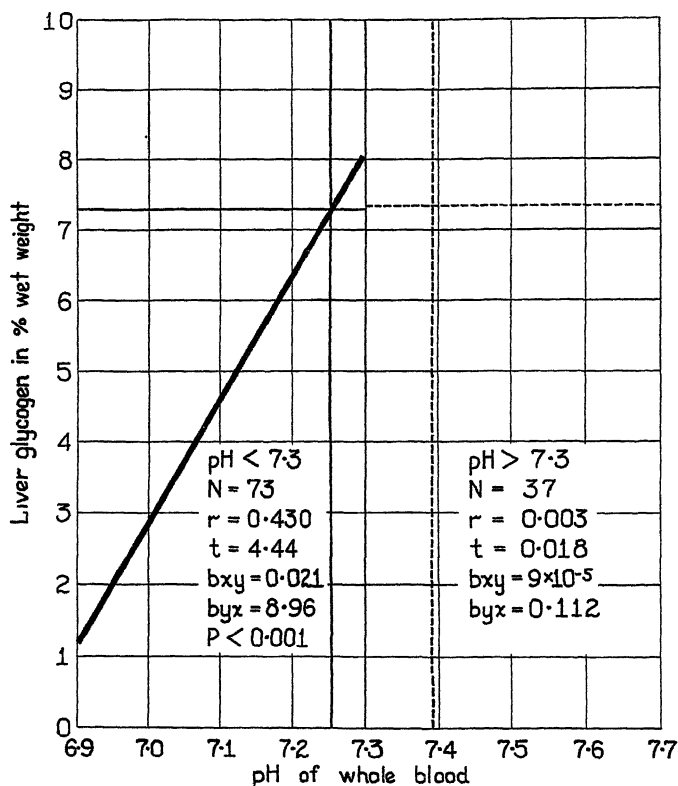


FIG. 1. Representation of the relationship between the liver glycogen concentration and the blood pH. The  $r$  line has been calculated for the pH up to and including 7.30. The pH and liver glycogen means are given for the higher pH ranges, but no relationship has been found.  $N$  = number of experimental animals.  $r$  = (correlation coefficient) =  $\Sigma xy / \sqrt{\Sigma x^2 \times \Sigma y^2}$ . The correlation coefficient may take any value between +1 and -1. If  $r$  is zero, the two factors are independent, while the nearer  $r$  approaches  $\pm 1$ , the greater the degree of correlation.  $t$  = test of significance of correlation coefficient in terms of the standard error. The probability may be obtained, when  $t$  and  $N$  are known, from Fisher's table.  $bxy$  and  $byx$  are required in the equations  $X = Mx + bxy(y - My)$  and  $Y = My + byx(x - Mx)$  which determine the locations of the regression curves.

shaft with oxalate solution. Just before the blood was drawn, the temperature of the syringe was adjusted to 38°.

When anesthesia was complete, the rat was placed in the supine position and the needle was forced between the lower ribs and then into the heart. As soon as the needle entered the heart, the blood flowed out through the hole in the needle. The first few drops had come into contact with air and were wasted. The index finger

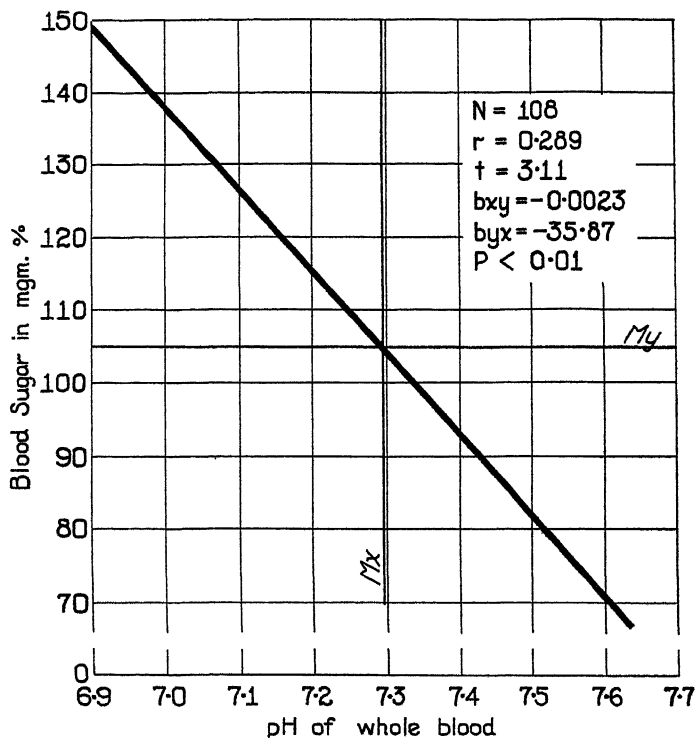


FIG. 2. Representation of the  $r$  line in the relationship between the blood sugar concentration and the blood pH. The symbols are explained in the legend to Fig. 1.

was then placed tightly over the sleeve hole and the plunger gently guided back as the blood flowed into the syringe.

After the blood had been drawn, the needle was removed from the syringe and the blood mixed by rotation of the syringe, with a finger held firmly over the tip. As soon as the blood sample had been taken, another operator removed the liver and muscle for the tissue determinations as has been previously described.<sup>1</sup>

The pH of the whole blood was determined immediately. A Leeds and Northrup universal pH potentiometer was used with the electrode system in a shielded, constant temperature air bath, maintained at  $38^{\circ} \pm 0.1^{\circ}$ . The glass electrode was a modified form of that designed by MacInnes and Belcher (12).

Immediately after the blood was mixed, the tip of the syringe was joined to the lower outlet of the electrode system by means of a short rubber tube and with the 5-way stop-cock open the blood was forced up through the electrode chamber. The first 1 cc. was passed on into the cup, so that this portion, which had come into contact with air, was discarded. The stop-cocks were then closed and the lower stop-cock turned to form the KCl bridge with the reference electrode.

About 2 minutes were allowed to permit the temperature of the blood to come into equilibrium with that of the electrode before a reading was taken. Several readings were taken over a period of 2 to 3 minutes. These usually checked with each other within the sensitivity of the instrument. The blood was then drained from the electrode and the electrode was rinsed eight to ten times with distilled water at  $38^{\circ}$  which was admitted through the cup at the top. Electrode asymmetry was checked with standard buffer solutions before and after each series of determinations.

We have induced modifications in the blood pH by intraperitoneal injections of isotonic solutions of sodium bicarbonate or ammonium chloride. Similar injections of isotonic sodium chloride were made in the control animals. In each case two injections of 5 cc. were given, the first 2 hours and the second 1 hour before the tissue samples were removed.

### *Results*

The deviations presented in Table I are indicative of considerable variation. The variability in liver glycogen may be partly the result of the necessary extra handling of the animals. The method, therefore, does not permit of precise experimental control of the pH or of the glycogen and blood sugar concentrations. However, we were enabled to study a fairly wide range of pH within what might be termed "normal" limits. The blood pH of each individual studied was plotted against its liver glycogen, muscle glycogen, or blood sugar concentration and the resulting scatter diagrams

were analyzed by the regression method (13). When the blood pH was plotted against the liver glycogen or blood sugar, indications of significant relationships, as measured by *t*, were found. The liver glycogen increases with the pH to about 7.3, after which changes in pH within the range studied appear to have little effect on the liver glycogen. The blood sugar, on the other hand, tends to vary inversely as the pH throughout the entire range studied. The data at hand give no indication that muscle glycogen changes are correlated in any way with the pH.

TABLE I

*Effect of Injections of Isotonic Solutions on Blood pH, Tissue Glycogen, and Blood Sugar*

Isotonic solution injected intraperitoneally	No. of rats	pH of blood	Glycogen in liver, wet weight	Glycogen in muscle, wet weight	Blood sugar
			<i>per cent</i>	<i>per cent</i>	<i>mg. per cent</i>
Ammonium chloride.	11	7.16 $\pm$ 0.04	5.93 $\pm$ 0.52	0.90 $\pm$ 0.03	121 $\pm$ 3
Sodium chloride....	17	7.29 $\pm$ 0.01	7.03 $\pm$ 0.23	0.95 $\pm$ 0.02	108 $\pm$ 2
“ bicarbonate	17	7.40 $\pm$ 0.02	6.90 $\pm$ 0.35	0.95 $\pm$ 0.04	106 $\pm$ 2

The deviations given are the deviation of the mean ( $\epsilon_M$ ).

### *Interpretation*

There is a definite relationship, as we have previously shown (11), between the character of the intraperitoneally injected solution and the pH of the blood. Rats injected with 10 cc. of isotonic ammonium chloride exhibit a significantly lower pH than those receiving isotonic sodium chloride. This depression has been shown to be present after the second injection and to persist for at least 5 hours. Similarly, intraperitoneal sodium bicarbonate appears to raise the blood pH by a significant amount during the same time interval. However, as one might expect, there is considerable variation in the pH values induced by these methods. The variations are probably influenced by several factors, among which may be mentioned the rate of absorption of the salts, the rate of excretion of the introduced ions, the effective buffering capacity of the body fluids, and compensatory respiratory changes.

Because of these pH variations it appeared best to compare the blood pH values with the carbohydrate values of each animal.

These relationships as measured by the regression method have already been indicated.

Since it is generally accepted that the blood pH affects the respiration and an increased acidity is associated with a lowered blood CO<sub>2</sub> content, the possibility was suggested that changes in the CO<sub>2</sub> concentration, apart from changes of pH, might have an influence upon the blood glucose and liver glycogen. In our experiments it has not been possible to demonstrate, however, that the blood CO<sub>2</sub> has any such effect. In the experiments in which the blood pH was experimentally altered the carbohydrate relationships were always more closely associated with pH changes than with variations in the blood CO<sub>2</sub> content. Furthermore, in experiments in which the blood CO<sub>2</sub> content was increased by maintaining the animals for 12 hours in atmospheres containing up to 10 per cent of carbon dioxide no change in the blood sugar or liver glycogen could be associated with the CO<sub>2</sub> content of the blood. This was true for both fasted and fed animals. However, these results of themselves do not prove whether it is the CO<sub>2</sub> content or the pH of the blood and tissues that is related to carbohydrate storage and mobilization.

The evidence from these experiments tends to substantiate Donnelly's thesis that the effect of the pH on the stability of glucose and glycogen is qualitatively the same *in vitro* and *in vivo*, although apparently the quantitative effect is slight. The low value of the coefficients of correlation would thus lead us to believe that although the pH may be a factor in controlling the liver glycogen and blood sugar it is usually only of minor importance.

Perhaps the marked changes in pH associated with the acidosis sometimes occurring in diabetes mellitus may be a contributing factor to the hyperglycemia and reduced liver glycogen storage. However, our work seems to indicate that it is doubtful whether this relationship is of much importance in the normal organism.

#### SUMMARY

An investigation of the effects of exogenously induced pH changes indicates that there is a correlation between the pH of the blood and the stored and mobile carbohydrate, although it is probable that in the normal rat this correlation is of relatively slight quantitative importance.

Statistical analyses give indications of the following relationships.

1. The liver glycogen tends to vary directly with the blood pH up to a pH of about 7.3. When the pH is higher than this, there is no apparent correlation.

2. The blood sugar tends to vary inversely with the pH throughout the pH range studied.

3. No relation between the blood pH and the concentration of muscle glycogen has been demonstrated.

We wish to express our sincere gratitude to Dr. E. L. Scott for his invaluable assistance throughout this study and to Dr. K. S. Cole for his suggestions in regard to the pH determinations.

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# PHOSPHORUS METABOLISM IN PHLORHIZIN DIABETES, WITH RADIOACTIVE PHOSPHORUS AS AN INDICATOR\*

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By ordinary analytical methods, only the amounts and changes in amounts of compounds in tissues are observable. By the use of the isotopes, it is possible to determine the rates at which chemical reactions proceed in the body and to demonstrate changes in rate which can be attributed to certain conditions (1-3). Considerable indirect evidence has accumulated in support of the hypothesis that the biological action of phlorhizin is due to a retardation in the rate at which some or all of the phosphorylation processes proceed in the body (4-7). Since this inhibition need not be accompanied by any change in the absolute or relative amounts of the phosphorus compounds in the tissues concerned, but only in the rate of phosphorus turnover, radioactive phosphorus presents itself as a useful tool for the examination of this theory.

It was of interest to determine (a) whether the glycosuria produced by phlorhizin involves an alteration in the phosphorus turnover of the blood and kidney; (b) whether the relationship between the kidney and blood phosphorus turnover is altered by the drug; and (c) in the light of the observations of Wertheimer (7) and Verzář (5) that phlorhizin inhibits the absorption of sugars and fat, whether there is an inhibition in the phosphorus turnover of the intestine.

In this study, the total phosphorus turnover of the blood, kidney,

\* The data in this paper were taken from the thesis presented by the author to the Graduate School of the University of Rochester in partial fulfilment of the requirements for the degree of Doctor of Philosophy.



and intestine and the phospholipid phosphorus turnover of the kidney and intestine of normal and phlorhizinized rats were compared.

### *Experimental Conditions and Methods*

*Phlorhizin Preparation and Injection*—Schering-Kahlbaum phlorhizin was purified according to the method of Deuel and Chambers (8). The melting point,  $173^{\circ}$ , of the resultant material was in close enough agreement with that quoted by Richter-Anschütz (9),  $171^{\circ}$ , to insure the reasonable purity of the preparation used.

The classical method of phlorhizin administration, subcutaneous injection of an olive oil suspension, is a messy and non-quantitative procedure; the suspension is far from uniform and likely to clog in the syringe needle, so that the dosage cannot be controlled with any reasonable degree of accuracy. Furthermore, since in this work the phospholipid phosphorus turnover was measured, the injection of considerable quantities of olive oil might have constituted a complicating factor. At the suggestion of Dr. R. R. Sealock of the Department of Vital Economics, propylene glycol was tried as a solvent. The glucoside dissolved rapidly and completely in the propylene glycol with the aid of slight warming. One such preparation, which contained 100 mg. of phlorhizin per cc. of propylene glycol, showed no signs of deterioration after standing in the laboratory for over 4 months. The glycol injections gave rise to no more local irritation than similar injections of olive oil, and, in the amounts administered (a single dose of 1 cc. or 0.25 cc. daily for 4 days), produced no toxic symptoms. This would seem to constitute a practical and quantitative method for the subcutaneous injection of phlorhizin.

*Treatment of Animals*—Thirty rats, ranging in weight from 130 to 208 gm., constituted a series of twenty phlorhizinized and ten control animals. They were divided into two groups in regard to phlorhizin administration; eight rats received 65 mg. of phlorhizin in 1 cc. of propylene glycol 1 to 2 hours prior to  $P^*$  administration; twelve rats received 70 mg. of phlorhizin in 1 cc. of propylene glycol *in toto* over a period of 4 days prior to the  $P^*$ ; the corresponding controls received the same amount of propylene glycol without phlorhizin. The  $P^*$  was given as a solution of  $Na_2HPO_4$  by stomach tube and was followed by 1 cc. of olive oil. The radio-

activity of the dose per animal ranged from<sup>†</sup> 10,000 to 45,000 counts per minute on our scale-of-four Geiger-Müller counter. Sixteen phlorhizinized and six control rats were killed 17 hours after the P\* feeding; the results on these animals are summarized in Table I. Four phlorhizinized and four control rats were killed 6 hours after P\* administration; the results for this group are also summarized in Table I.

*Preparation of Samples for Analysis*—The tissues studied were weighed as soon after removal from the body as possible. Phospholipid was prepared from one aliquot (10), and its amount and P:P\* ratio determined. Another sample of the tissue was ashed; the acid solution of the ash was analyzed for its P:P\* ratio and phosphorus content (11). The intestines were split open, washed, minced, weighed, and aliquots taken for lipid extract and for whole ash.

In a few cases, a further fractionation of the kidney phosphorus was carried out. One kidney was ashed in the usual manner; the other was ground in the cold and extracted five times with cold 5 per cent trichloroacetic acid. The filtrate was designated as the acid-soluble phosphorus, and its P:P\* ratio and phosphorus content determined. The phospholipid was prepared from the trichloroacetic acid residue in the usual manner.

The urine excreted by the individual animals during the period following the phosphorus administration was collected. To establish the degree of glycosuria, quantitative glucose determinations were made, in some cases, on aliquots by the method of Shaffer and Hartmann (12).

The degree of radioactivity of the samples was determined by the technique of Bale *et al.* (13). Since the phosphorus content of the blood, kidney, and liver was constant, results are expressed as the per cent of the original dose per gm. of tissue. In other cases there was appreciable variation, so that results are expressed as the per cent of the original dose per gm. of phospholipid, per mg. of acid-soluble phosphorus, and per mg. of intestinal phosphorus, respectively.

### Results

This work was undertaken in an effort to determine the extent to which phlorhizin poisoning influenced the phosphorus metabolism of the blood and kidney. The experimental period of

TABLE I

*Mean Values for Phosphorus Turnover in Rats 17 and 6 Hours after Administration of Radioactive P*

	Controls (17 hrs.)			Phlorhizinized (17 hrs.)		
	No. of rats	Range	Mean $\pm$ average deviation	No. of rats	Range	Mean $\pm$ average deviation
Urine excreted, cc.....	6	1.4 -11.5	3.8 $\pm$ 2.8	16	2.4 -18.0	8.2 $\pm$ 3.8
Per cent dose excreted.....	2	8.7 -11.9	10 $\pm$ 1.6	4	8.5 -15.6	11 $\pm$ 2.2
Per cent dose per gm. kidney phospholipid.....	5	9.8 -15.2	14 $\pm$ 2.4	15	9.6 -17.0	14 $\pm$ 1.8
Per cent dose per gm. kidney.....	6	0.73- 1.01	0.9 $\pm$ 0.1	16	0.50- 1.09	0.8 $\pm$ 0.12
Per cent dose per mg. kidney acid-soluble phosphorus....	2	0.48- 0.64	0.6 $\pm$ 0.08	4	0.45- 0.69	0.6 $\pm$ 0.07
Per cent dose per gm. blood	6	0.09- 0.24	0.17 $\pm$ 0.03	13	0.09- 0.21	0.12 $\pm$ 0.03
Per cent dose per gm. intestinal phospholipid.....	2	10.1 -22.6	16 $\pm$ 6.2	6	10.3 -15.8	13 $\pm$ 1.6
Per cent dose per mg. intestinal phosphorus..	2	0.21- 0.31	0.3 $\pm$ 0.02	6	0.22- 0.41	0.3 $\pm$ 0.04
Per cent dose per gm. liver phospholipid.	2	14.3 -18.6	16 $\pm$ 2.1	6	10.8 -20.2	17 $\pm$ 3.3
Per cent dose per gm. liver..	2	1.0 - 1.1	1.1 $\pm$ 0.05	6	0.66- 1.1	0.9 $\pm$ 0.11
	Controls (6 hrs.)			Phlorhizinized (6 hrs.)		
	No. of rats	Range	Mean $\pm$ average deviation	No. of rats	Range	Mean $\pm$ average deviation
Urine excreted, cc.....	4	0.5 - 4.8	2.4 $\pm$ 1.6	4	4.5 - 9.0	6.7 $\pm$ 1.7
Per cent dose excreted.....	2	15.3 -16.3	16	2	13.7 -14.2	14

TABLE I—*Concluded*

	Controls (6 hrs.)			Phlorhizinized (6 hrs.)		
	No. of rats	Range	Mean $\pm$ average deviation	No. of rats	Range	Mean $\pm$ average deviation
Per cent dose per gm. kidney phospholipid. . . . .	4	7.4 -13.6	10 $\pm$ 1.5	4	8.2 -15.2	12 $\pm$ 2.3
Per cent dose per gm. kidney. . . . .	4	0.69- 1.31	1.1 $\pm$ 0.2	4	0.82- 1.36	1.0 $\pm$ 0.2
Per cent dose per gm. blood	4	0.12- 0.20	0.16 $\pm$ 0.03	4	0.11- 0.21	0.15 $\pm$ 0.03
Per cent dose per gm. intestinal phospholipid. ....	2	13.0 -13.1	13	2	14.1 -14.8	14.5
Per cent dose per mg. intestinal phosphorus. ....	2	0.33- 0.47	0.4 $\pm$ 0.07	4	0.43- 0.63	0.51 $\pm$ 0.06

17 hours was chosen because it is well within the period during which phlorhizin is effective in producing glycosuria and inhibiting fat absorption (7) and because it is near the peak for the incorporation of P\* into the renal phospholipid (1). When it was shown that at 17 hours the phosphorus turnover of the phlorhizinized rats did not differ from that of the controls, a few animals were used with an experimental period of 6 hours. In this case, also, no difference was demonstrated.

*Urine*—In every case the phlorhizin produced a diuresis, which was marked, although extremely variable. The average excretion of the 17 hour controls was 3.8 cc.; that of the 17 hour phlorhizinized rats was 8.2 cc. (Table I). The 6 hour controls excreted on the average 2.4 cc., while the corresponding phlorhizinized rats excreted 6.7 cc.

None of the control urines gave a positive test for reducing sugar with Fehling's reagent, while every phlorhizinized rat gave evidence of glycosuria. Two phlorhizinized rats excreted 314 and 331 mg. of sugar in 6 hours; the urines of the corresponding controls were glucose-free.

There was no significant difference in the degree of radioactivity of the urines of the control and phlorhizinized rats; the average control excretion for two animals was 10 per cent in 17 hours; that of the four corresponding phlorhizinized rats was 11 per cent. Two 6 hour controls excreted 16 per cent of the dose; the corresponding experimental animals 14 per cent.

*Kidneys*—Metabolism of the kidney phospholipid phosphorus appeared to be unaltered by phlorhizin poisoning; in the 17 hour series, the mean per cent dose per gm. of renal phospholipid for the control and phlorhizinized animals was the same, 14. In the 6 hour series, the control and experimental values were 10 and 12, respectively; this difference is within the normal physiological range and cannot be considered as significant.

The study of the phosphorus turnover of the whole kidney revealed no significant difference between the control and phlorhizin-poisoned rats. In the 17 hour series, the control and experimental values for per cent dose per gm. of kidney were 0.9 and 0.8, respectively; this difference of 12 per cent is not significant. The control value for the 6 hour group, 1.1, is in excellent agreement with the corresponding experimental value, 1.0.

The average per cent dose per mg. of acid-soluble phosphorus, 0.6, was identical for the few control and phlorhizinized rats studied.

*Blood*—Only in the blood was there an appreciable difference in the phosphorus turnover in the direction assumed; *i.e.*, a lower value for the control animals. In the 17 hour series, the control and experimental values for the per cent dose per gm. of blood were 0.17 and 0.12, respectively, a difference of 29 per cent, the significance of which, in the light of the large average deviation, is very doubtful. The lack of significance of this difference is borne out by the close agreement found between the values for the control and experimental groups, 0.16 and 0.15, respectively, in the 6 hour series.

*Intestine and Liver*—Because of the importance of the phosphorylations which occur in the intestine and liver, the phosphorus metabolism of these organs was studied in the case of a few phlorhizinized and control rats. As Table I indicates, the phosphorus turnover in liver and intestine was not affected by phlorhizin poisoning.

In none of the tissues studied did phlorhizin produce any significant change in the phospholipid and phosphorus content, or in the  $P^*:P$  ratio of the phospholipid and phosphorus.

#### DISCUSSION

The theory that the biological action of phlorhizin is due to the inhibition of phosphorylation was built up largely from the following observations: Wertheimer (7), Lundsgaard (4), and others found that the glucoside abolishes the selective absorption of the hexoses from the intestine; this preferential absorption was presumed to be due to phosphorylation of the hexoses in the gut mucosa. Verzár and Laszt (5) demonstrated a retardation of fat absorption in phlorhizin poisoning, a process which has been shown by Sinclair (14) to involve phosphorylation of the fatty acid in the mucosa. Lundsgaard (4), among others, showed that phosphorylation *in vitro* in various systems is retarded by the glucoside. These data indicate that the phosphorus turnover of the phlorhizinized and normal rat is essentially the same, and, therefore, that phlorhizin in the intact animal does not act by an inhibition of phosphorylation processes.

Complete fractionation of the phosphorus compounds was not attempted in this work owing to the amounts of tissues and doses of  $P^*$  available. Since the relevant evidence, in line with the present conception of the biological oxidation-reduction systems and the breakdown of muscle glycogen to lactic acid, indicates that the biological phosphorylations are interdependent, an inhibition in the rate of phosphorus turnover of one system should be reflected in the tissue as a whole and in other systems. Since this was not the case, phlorhizin must act in some way other than by the inhibition of phosphorylation processes.

#### SUMMARY

1. A new method for the subcutaneous injection of phlorhizin was described. Phlorhizin dissolves readily in propylene glycol which, in the doses given, produced no toxic symptoms.

2. A comparison was made of the phosphorus turnover in certain tissues of phlorhizinized and normal rats, 6 and 17 hours after the administration of radioactive phosphorus. No differences were demonstrable at either time in the rate of incorporation of

the P\* into the kidney, intestine, blood, and liver, or into the renal, intestinal, or hepatic phospholipid of the normal and phlorhizinized rats. Neither was there any difference in the urinary excretion of P\*, although the phlorhizinized rats showed marked diuresis and glycosuria.

3. Contrary to the hypothesis that the biological action of phlorhizin is due to the retardation of phosphorylation, it was concluded that phlorhizin does not inhibit phosphorylation processes in the intact animal.

The author wishes to express her appreciation to Dr. W. R. Bloor and to Dr. H. C. Hodge for their advice and interest in this work; to Dr. W. F. Bale and Mr. John Bonner of the Department of Radiology for the building and maintenance of the Geiger-Müller counters; and to Dr. S. N. Van Voorhis of the Department of Physics for radioactive phosphorus.

*Addendum*—Since this paper was written Rapoport, Nelson, Guest, and Mirsky (15) have reported a decrease in the phosphorus turnover of the pyrophosphate fraction of the kidney inorganic phosphorus due to phlorhizin poisoning.

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## THE ESTIMATION OF SMALL AMOUNTS OF QUININE IN BLOOD AND OTHER BIOLOGICAL MATERIALS

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The literature contains various methods for the estimation of quinine in blood, all of which depend on similar principles (1-3). These, in general, involve extraction of the blood with an immiscible solvent (ether) with subsequent determination by some nephelometric or colorimetric procedure. The most practicable procedure appears to be that recommended by Vedder and Masen (4). These authors recommend both a nephelometric and a colorimetric procedure, of which the former, in which the turbidity of a suspension of quinine silicotungstate is utilized, appears preferable.

In the Vedder and Masen nephelometric method the sample of blood, dried on a pad of asbestos or strips of filter paper, is extracted with ether. The ether extract is evaporated to dryness and the residue taken up with 0.5 N HCl and filtered if necessary. The quinine is precipitated as the silicotungstate and compared nephelometrically with an appropriate standard.

This method, in the hands of the present authors, appeared to be open to certain objections to be described below but to offer the most promise as a starting point. We have therefore made a critical study of its various steps in order to obtain a method of maximum simplicity and accuracy for use in a program of investigation of the mechanism of quinine absorption. To this end, it was also desired, if possible, to take advantage of the speed and simplicity of the photoelectric colorimeter, used as a nephelometer, since visual nephelometric readings quickly produce great eye fatigue with consequent inaccuracy, superimposed on the other inaccuracies of the method. Since the photoelectric



colorimeter has received comparatively little attention as regards its adaptability to nephelometric methods, a detailed investigation of this phase of the method was necessary.

The instrument used was the Evelyn macro-micro photoelectric colorimeter<sup>1</sup> with an appropriate light filter as originally described by Evelyn (5). The adaptation of this instrument and the modifications of the original Vedder and Masen method, which, for various reasons, were found necessary, are described below.

This procedure, described under "Method," has been successfully used on some preliminary studies of quinine absorption in dogs. It is intended to continue these studies on both animals and human subjects.

#### EXPERIMENTAL

*Extraction Procedure*—Preliminary estimations of quinine in blood by the Vedder and Masen method, with a visual nephelometer, indicated considerable difficulty in obtaining complete removal of the quinine from the sample of blood dried on either asbestos or filter paper. For this reason we have adopted a continuous extractor in which the ether is slowly passed in droplets through the oxalated sample. The ether layer continuously overflows into the bottom flask. This extractor, a modification of that first described by Clausen (6), is shown in Fig. 1. It has been made up in various sizes. In the selection of a heater for these extractors, incandescent lamps, sometimes used for low temperature distillation, should be avoided. The sensitivity of small amounts of quinine to light at the temperature of boiling ether is such as to cause losses of several per cent in the recoveries of quinine.

As a result of a series of experiments in which the time of continuous extraction was varied, a 4 hour period is recommended. The 2 hour periods of extraction used by Vedder and Masen have resulted in yields of less than 85 per cent by our own procedure and still less by the use of the asbestos tube recommended by the above authors.

*Optimum pH for Extraction*—Vedder and Masen state that the normal pH of blood is sufficiently alkaline to render the

<sup>1</sup> Supplied by the Rubicon Company, Ridge Avenue at 35th Street, Philadelphia.

quinine soluble in ether. Our own work confirms this conclusion. The two dissociation constants reported by Christophers (7) (pK values of 5.70 and 9.84) for quinine indicate that at pH 7.4

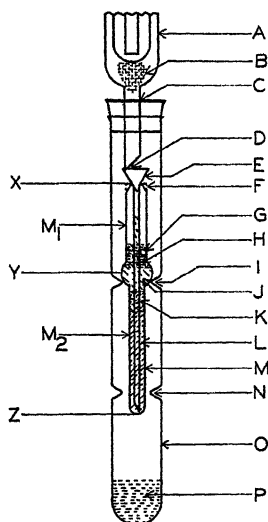


FIG. 1. Extraction apparatus. The parts and assembly of the extractor unit as employed for the extraction of a sample of blood or urine are described as follows: A, Hopkins condenser; B and H, tufts of fiber glass; C, hole in cork stopper bored slightly off center; D, tip of condenser touching side of funnel; E, 20 mm. funnel with a stem 180 mm. in length and 4 mm. outside diameter (used interchangeably with sample tubes constructed for 2, 5, and 10 ml. samples); F, punch marks for the support of the funnel; G, overflow for ether, 4 mm. in diameter; I and N, punch marks 190 mm. and 105 mm. respectively from the bottom of the extractor jacket for support of the sample tube; J, ether layer; K, ether-blood emulsified zone; L, sample of blood or urine; M, sample tube (dimensions: from X to Y, 70 mm.; from Y to Z, 110 mm.; outside diameter 16 mm. at  $M_1$ , 22 mm. at Y, and 9, 12, and 16 mm. respectively at  $M_2$  for 2, 5, and 10 ml. sample tubes); O, extractor jacket 320 mm. in length and 32 to 35 mm. outside diameter; P, ether reservoir, 15 to 20 ml. The scale drawing of the apparatus shows a sample tube for a 5 ml. sample; in this and the other sample tubes, the proper sample volume stands in a column 80 to 85 mm. high when the funnel stem filled with ether is in place.

only about 11 per cent exists in the form of the free base. However the buffer capacity of the blood insures a continuous shift of the quinine from salt to base as the extraction proceeds and the

end-result is a practically complete extraction. (See the discussion of total recoveries below.)

Because of its lower buffer capacity it has been found necessary to bring the pH of urine to 9.0 to 10.0 (phenolphthalein) before extraction.

*Purity of Ether*—At an early stage in this work it was observed that the use of certain lots of ether for the extraction of buffered solutions of quinine produced a light brown solution when the quinine was taken up in 0.03 N HCl. In such cases erratic recoveries were obtained. Shaking of these lots of ether with 10 per cent NaOH followed by redistillation produced a sample with which the brown color and poor recoveries were no longer obtained. As a precautionary measure, before distillation the ether should be entirely freed from peroxides by continuous washing with the sodium hydroxide. Peroxides may be detected by washing a small sample of ether from the separatory funnel with water until neutral and then testing the ether for peroxides by the ferrous ammonium sulfate-ammonium thiocyanate method. Ether thus purified has given satisfactory results after having been stored in 1 liter brown glass bottles containing a steel wire in the refrigerator for as long as 3 months. We have found that routine purification of all new lots of ether by the above procedure is advantageous.

The development of the brown color in the acid solution in cases in which untreated ether was used appears to be coincident with a positive peroxide test. In several instances samples of ether, which gave slight tests for peroxides and a brown color in the acid solution when buffered solutions were extracted, gave no coloration in the acid solution when blood was extracted. This may have been due to the destruction of the interfering substances by reducing agents in the blood. An unsuccessful attempt was made to extract blood with a sample of ether which gave a heavy peroxide test. When this ether came in contact with the blood, a granular, chocolate-colored precipitate, the color of which was probably due to methemoglobin, formed almost immediately and clogged the extractor.

*Evaporation of Ether*—While too vigorous evaporation of the ether solution is to be avoided because of mechanical losses, we have found that the practice of allowing it to evaporate spon-

taneously by standing for some hours at room temperature or overnight is even more objectionable. In the latter case the quinine is often oxidized to products which give a brown solution in the hydrochloric acid and which give very low yields of the silicotungstate. We recommend, therefore, placing the extractor jacket in a bath at 55°.

*Concentration of Hydrochloric Acid*—Quinine silicotungstate turbidities prepared from solutions containing constant amounts of quinine (3.0 mg. per liter) in concentrations of hydrochloric acid graded from 0 to 0.5 N HCl were each compared in the visual nephelometer, with the turbidity produced in 0.5 N HCl as the standard, since this concentration of acid is used by Vedder and Masen. Preliminary observations showed that a decrease in the acid concentration below 0.5 N produced a slow increase in the apparent quinine concentration to about 0.15 N acid, followed by a rapid increase to a maximum at from 0.025 to 0.035 N acid and a rapid decrease from this point to zero normality. The apparent concentration at the maximum was approximately 50 per cent above that observed in 0.5 N acid. More detailed studies by the same procedure confirmed these results.

Further confirmation was obtained with the Evelyn photoelectric colorimeter on a similar series of solutions and data collected from a minimum of four triplicate sets of samples on each solution. Plotting the average galvanometer reading against acidity, one obtains Curve A in Fig. 2. This quinine silicotungstate curve is similar to the nicotine silicotungstate curve obtained by Spies (8) except that the minimum solubility, in the case of nicotine, is at 0.005 N HCl. It is obvious that the minimum solubility of the quinine silicotungstate occurs at 0.03 N HCl and this concentration would therefore be expected to yield a much more delicate method.

*Filtration*—In the Vedder and Masen method filtration of the 0.5 N HCl solution of the extracted quinine through Whatman No. 42 paper is recommended to remove any turbidity. In view of the possible absorption of quinine on the filter paper, the advisability of this step seems questionable. To test this point, standard 12 ml. samples in triplicate containing equal concentrations of quinine (3 mg. per liter) in varying concentrations of hydrochloric acid were filtered by gravity through Whatman No.

42 fluted paper and from each filtrate a 10 ml. sample was taken for comparison of quinine concentration with the unfiltered standards, both by nephelometric and photelometric methods. The results of both series are similar and those of the latter are shown in Curves B and C, Fig. 2.

It is obvious that in from 0.0 to 0.5  $N$   $HCl$  the quinine can in no case be filtered through paper at room temperature without loss. This loss is very small and at a minimum at 0.03  $N$   $HCl$ ; at 0.5  $N$  it is considerable and is at a maximum at zero acidity.

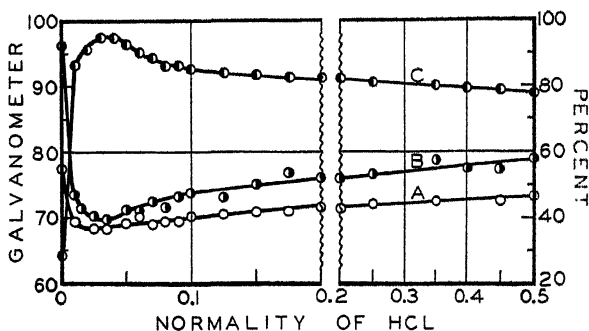


Fig. 2. Adsorption of quinine on filter paper and solubility of its silicotungstate in solutions of variable acidity. The ordinate values for Curve A and Curve B are defined on the left and for Curve C on the right. The abscissa values are common to all the curves. Curve A describes the quinine silicotungstate prepared from standards containing 3 mg. of quinine per liter in different concentrations of  $HCl$ ; Curve B, the same except that the quinine standards were filtered through Whatman No. 42 paper before preparation of the silicotungstate. Curve C represents the ratio of apparent quinine concentrations in filtered and unfiltered standards when compared by the macro curve in Fig. 5. All observations were made with Filter 400.

Various filters have been tried for the quantitative filtration of quinine in 0.03  $N$   $HCl$  solution at room temperature. A comparison of the filtrates with corresponding unfiltered solutions showed the filtrate, from each of the filters, to contain the following percentages of the quinine in the unfiltered solution: 107, Arthur H. Thomas No. 5160; 110, Delta No. 377; 88, Reeve-Angel No. 202; 85, Eaton and Dikeman No. 613; 94 to 100, Whatman No. 42;  $100 \pm 2$ , funnel stem packed with Pyrex fiber glass No. 719 or Jena glass crucible No. 4.

It should be noted that Vedder and Masen recommend filtration while the solution is hot, whereas the curves in Fig. 2 were obtained by filtration at room temperature. The rapidity with which a few ml. of solution cool under these conditions probably makes the above experiment at room temperature applicable. However, as a further test of their conditions we have compared the concentration of quinine standards in 0.5 N HCl with numerous samples of the same solution filtered by gravity through Whatman No. 42 fluted paper immediately after removal of the sample from a boiling water bath. The filtrates contained 86 per cent of the original in a 6 mg. per liter standard and 75 per cent of the original in a 3 mg. per liter standard; the latter is similar to results obtained by using the same filter at room temperature, as shown by Curve C, Fig. 2.

*Ratio of Reagent to Quinine*—In the procedure recommended, 0.2 ml. of 10 per cent silicotungstic acid is used for 10 ml. of the quinine solution. For quinine concentrations of 10 mg. per liter the molar ratio of reagent to quinine is 20:1. This amount of reagent, adopted from the Vedder and Masen procedure, has been used throughout and should be strictly adhered to, since the reagent is an acid as well as a precipitant for quinine and variation in the amount used will produce variation in the total acidity of the solution.

*Quinine Standards*—Pure quinine standards, whether for comparison in the visual nephelometer or for the establishing of a standard reference curve for the photometric method, were prepared as follows: Merck's U.S.P. quinine sulfate was recrystallized three times from water slightly acidified with sulfuric acid and the crystalline product, dried to constant weight in a vacuum desiccator over phosphoric anhydride, was used for the preparation of all quinine standards. The purity of the quinine sulfate was confirmed by optical rotations which were determined on portions of dried crystals from each crystallization (9). A stock solution of quinine sulfate containing 200 mg. per liter of quinine (as free base) in 0.03 N HCl was used for the preparation of a series of standards by dilution with the appropriate amounts of 0.03 N HCl. Standards in other concentrations of acid were similarly prepared.

It is frequently stated that quinine standards in dilute aqueous

solutions are unstable to light but can be stored in dark bottles exposed to ordinary laboratory conditions. Quinine standards containing 3 mg. per liter in 0.5 N HCl and in 0.03 N HCl each in clear bottles and in amber bottles were placed in a dark cabinet, in diffused light, and in direct sunlight. Determinations by the photelometric method described below were made over a period of 4 months.

The results showed that the precautions usually advised are fully justified. Under all conditions, standards in 0.03 N HCl were more stable than those in 0.5 N HCl. Standards in amber bottles were more stable than those in clear bottles and, in all cases, the greater the exposure to light the greater the apparent loss. For example, the standard in 0.5 N HCl exposed in a clear bottle to direct sunlight for 1 day suffered an apparent loss of 40 per cent. Similar results were obtained with standards of other concentrations of quinine. Standards in 0.03 N HCl in amber bottles, under ordinary laboratory conditions, have remained stable for 6 months. More detailed studies of this decomposition will be reported later.

*Effect of Certain Physical Factors in Preparation of Quinine Silicotungstate*—The procedure required for preparation of the quinine silicotungstate involves the following steps, (a) addition of the silicotungstic acid, (b) heating, (c) cooling, with formation of the precipitate. Since the properties of the resulting turbidity are likely to be determined by the conditions of its preparation, each of the above steps was separately investigated on both macro and micro samples according to the final procedure described below. Steps (b) and (c) in particular required comparison by both procedures, since the latter calls for different quantities of material and the rate of heating and cooling of the sample is necessarily different.

The quinine samples, after addition of the reagent, were allowed to stand at room temperature for varying periods of time up to 40 minutes before being heated without any significant differences in the final result.

The second step, that of heating the sample in a boiling water bath, is most simply carried out by suspending a wire cage, holding the sample tubes, in the boiling water to a depth slightly greater than that of the samples in the tubes.

Variations in the time of heating of macro samples between 3 and 10 minutes caused no appreciable change in recoveries from 3 mg. per liter standards. Heating periods of less than 3 minutes caused greatly lowered recoveries. With no heating, the above standards produced no turbidity; more concentrated standards produced very little. In the case of micro samples, with which small volume permits more rapid attainment of temperature, heating periods of from 1.5 to 2.5 minutes gave satisfactory recoveries. Beyond the above limits lower values were obtained. The period of heating was therefore standardized at 5 minutes for macro and 2 minutes for micro samples. Under these conditions the same standard solution gives identical values by either procedure.

The above studies on the time of heating were carried out with a constant cooling period of 5 minutes in all cases. The cooling bath consists of containers similar to those used for heating and the rack holding the tubes is transferred from the one to the other. Tap water, running freely through the bath, is most convenient but under summer conditions a bath cooled to  $20^{\circ} \pm 2^{\circ}$  must be used. Higher cooling temperatures cause incomplete precipitation of the quinine silicotungstate; a series of samples, heated under the above standard conditions and cooled in tap water at  $30\text{--}32^{\circ}$  gave results 6 to 8 per cent low when compared with the same standards cooled at  $20^{\circ}$ . A cooling period for both macro and micro samples of 5 minutes in running water at  $18\text{--}22^{\circ}$  has therefore been adopted.

*Stability of Quinine Silicotungstate*—The stability of the quinine silicotungstate prepared from standards of from 1 to 20 mg. of quinine per liter was investigated in both 0.03 N and 0.5 N HCl. In each case photelometric readings were taken at various intervals from zero time (completion of cooling) to 90 minutes. The results are summarized in Fig. 3 in which galvanometer readings at zero and at 90 minutes are plotted against the concentration of quinine in both acidities. It is obvious that at the lower acidity, the zero and 90 minute curves are practically coincident, whereas in 0.5 N acid the time effect is much greater. Moreover, since the time effect in 0.5 N acid varies considerably with the concentration of quinine, a further error is introduced when one uses the Vedder and Masen procedure which calls for 0.5 N HCl unless the standard



and unknown are of practically identical concentrations or the time interval between cooling and reading is practically zero.

Further study of the stability of quinine silicotungstate with variation in acid concentration showed a maximum and constant stability from zero to almost 0.04 N acid, a decrease to a minimum at about 0.125 N acid followed by a slow rise as the 0.5 N concentration is approached. However, in no case does the stability at 0.5 N acid equal that at 0.03 N.

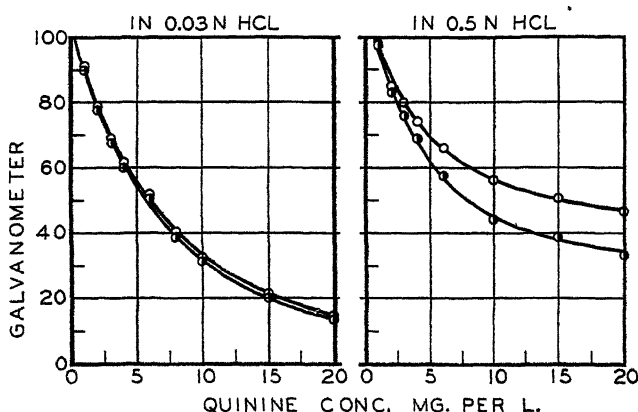


FIG. 3. Stability of quinine silicotungstate in 0.03 N and 0.5 N HCl. Galvanometer readings at zero time (end of standing period), observed when quinine silicotungstate prepared from standards of different quinine concentrations in both 0.03 N and in 0.5 N HCl is examined in the photoelectric colorimeter, are plotted on the lower curve in each section of the figure. Readings on the same, 90 minutes later, are plotted in the upper curve of each section.

*Selection of Light Filter*—The absorption of light by quinine silicotungstate, quinine, and silicotungstic acid was studied by means of the Coleman regional spectrophotometer (30 m $\mu$  slit) which gave the data shown in Fig. 4.<sup>2</sup> As was to be expected, the curve is non-specific and the transmittance increases with increasing wave-length.

With Filters 400, 420, and 440 in the Evelyn photoelectric

<sup>2</sup> Special thanks are due to Dr. G. L. Donnelly of the Department of Pharmacology of this Medical School for the loan of the Coleman regional spectrophotometer and the visual nephelometer used in this study.

colorimeter these results were confirmed. In the macro colorimeter Filter 400 is recommended.

In the selection of a filter for the micro colorimeter another factor may influence the choice. The use of the very dense Filter 400 limits the galvanometer scale to about one-third its length. Filters 420 and 430-M are next in order of preference and with either of these filters the whole scale of the galvanometer may be

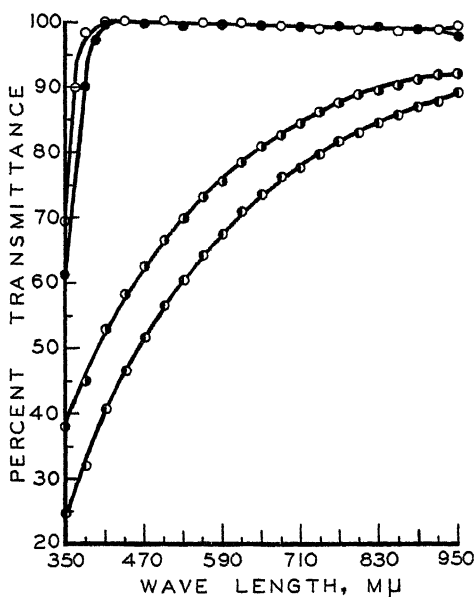


FIG. 4. Spectral transmittance of quinine, silicotungstic acid, and quinine silicotungstate. Silicotungstic acid O, quinine ●, quinine silicotungstate prepared from a standard containing 4 mg. of quinine per liter in 0.03 N HCl ●, 10 mg. of quinine per liter in 0.03 N HCl ○.

used, making possible a more accurate galvanometer reading. The No. 400 filter and shorter scale were chosen, however, because of the steeper concentration curve and, on the basis of numerous determinations, the results were more consistent.

*Standard Concentration Curves*—Numerous triplicate determinations were made by the technique described below on a number of quinine standards ranging in concentration from 1 to 20 mg. per liter. Calculation of the  $K$  value according to the Lam-

bert and Beer laws shows that a logarithmic relationship does not obtain over any sufficiently wide range of concentration. This necessitates using standard curves of galvanometer reading *versus* concentration of quinine. These curves are shown in Fig. 5. It should be noted that the abscissa values in Fig. 5, which apply equally to the macro and micro curves, refer to the concentration

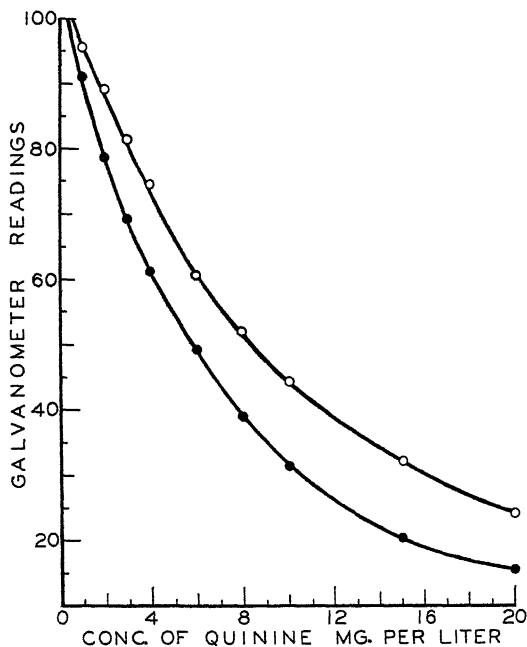


FIG. 5. Concentration curves for the macro- and microphotometric estimation of quinine as the silicotungstate. The points plotted as solid circles describe data obtained by the macroprocedure and those as open circles, by the microprocedure. All standards were prepared in 0.03 N HCl and all data were obtained with Filter 400.

of quinine in the original solution only when the residue from the ether evaporation is made up to the *same volume* as that originally taken for the analysis. Otherwise, the values read from the curve require multiplication by the appropriate factor.

The limits of accuracy of the determination of quinine in 0.03 N HCl are indicated in Table I. These data were obtained by determinations on several triplicate sets of samples of each of the

quinine concentrations listed in the second column of Table I. In succeeding columns are listed for both procedures the figures

TABLE I

*Error in Estimation of Quinine by Macro- and Microprocedures*

The macro- and microprocedures are compared at each concentration listed in the second column; observations on the macroprocedure are recorded in the first line and on the microprocedure in the second line at each concentration.

No. of triplicate analyses	Concentration of quinine		Galvanometer reading* †	Probable error in average of single triplicate	
	Actual	Observed*			Galvanometer reading
	mg. per l.	mg. per l.		mg. per l.	
9	1	0.87- 1.07 (1.00)	89.9-93.4 (91.1)	0.036	0.63
9		1.10- 1.46 (1.16)	93.6-96.3 (95.8)	0.070	0.53
10	2	1.85- 2.13 (2.00)	77.4-81.0 (79.0)	0.043	0.55
13		1.57- 2.57 (1.99)	85.0-92.6 (89.2)	0.154	1.14
6	3	2.81- 3.15 (3.02)	67.6-70.8 (69.3)	0.063	0.61
5		2.82- 3.54 (3.02)	77.5-83.0 (81.4)	0.160	1.21
6	4	3.89- 4.18 (4.02)	60.0-62.1 (61.2)	0.065	0.47
5		3.58- 4.24 (3.96)	72.8-77.3 (74.6)	0.134	0.98
6	6	5.84- 6.09 (6.00)	48.8-50.3 (49.4)	0.052	0.31
5		5.84- 6.21 (6.04)	60.0-62.3 (60.8)	0.080	0.43
6	8	7.79- 8.15 (8.06)	38.8-40.3 (39.1)	0.087	0.37
5		7.77- 8.07 (7.98)	51.8-53.0 (52.2)	0.066	0.26
6	10	9.60-10.29 (9.99)	30.9-33.0 (31.8)	0.147	0.48
5		9.89-10.15 (10.03)	44.3-45.0 (44.6)	0.067	0.21
6	15	14.18-15.42 (14.91)	19.9-21.8 (20.8)	0.298	0.44
5		14.56-15.66 (15.00)	31.0-33.0 (32.2)	0.248	0.45
3	20	19.22-20.57 (19.98)	15.5-16.3 (15.9)		
5		19.67-20.13 (19.99)	24.0-24.7 (24.2)	0.112	0.11

\* The range of observed values in the third and fourth columns is indicated by listing minimum and maximum results; parenthetically, the arithmetical mean of all the observations is recorded.

† In the microprocedure, the galvanometer readings are the result of multiplying the actual readings by the factor 3 or 4, since either one-third or one-fourth, respectively, of the whole galvanometer scale was used in all microanalyses.

for the extremes encountered and (in parentheses) the average of all triplicates, followed by the probable error of single triplicate determinations.

The method, when applied to blank samples of blood, has always given values corresponding to less than 0.5 mg. of quinine per liter. Extrapolation of the concentration curves of Fig. 5 towards zero concentration shows that this value represents the minimum which can be determined and that blank values may be regarded as indicating zero quinine concentration.

*Recoveries of Quinine from Blood and Urine*—Table II shows the recovery figures obtained on samples of blood and urine to which

TABLE II  
*Recovery of Quinine from Blood and Urine*

Quinine added	Quinine recovered					
	From blood*				From urine†	
	No. of extractions	Range	Average	Probable error of single recovery from average	At pH 7.4	At pH 9.0-10.0
$\gamma$		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
10	14	72-97	83	5.0		
20	7	87-100	94	3.0	94	110
30	7	90-105	96	3.1		
40	11	90-102	95	2.4	102	106
60					104	104
80					96	99
250					88	96
500					85	92
Average.....			92	3.4	95	101

\* The added quinine was placed in a 5 ml. sample of blood for each extraction and the extract was analyzed by the microprocedure.

† The added quinine was placed in a 10 ml. sample of urine and the extract was analyzed by the macroprocedure. The recovery data on urine represent single extractions at different quinine levels and pH.

varying amounts of quinine were added. The amounts were of the order of those most commonly encountered after administration of quinine by mouth (2 to 8 mg. per liter in blood and 2 to 50 mg. per liter in urine). It is obvious that considerable loss occurs at the 2 mg. level in blood, whereas with the larger amounts very satisfactory recoveries are obtained. These results would indicate that the method can be satisfactorily used in studies of the mechanism of quinine absorption and excretion. Comparison of

the apparent quinine content of normal urines from non-smokers *versus* moderate smokers showed, by our method, no indication of any interference from moderate amounts of nicotine (10).

### *Method*

*Extraction of Quinine from Blood or Urine*—Assemble the extractor as shown in Fig. 1 and pipette the blood or urine samples (the latter neutralized to pH 9 to 10) through the funnel. Add ether slowly through the funnel until about 15 ml. have overflowed into the extractor jacket. Introduce all liquids (sample and ether) into the sample tube through the funnel, so that the liquid flows down the side of the funnel instead of dropping freely and thus entrapping bubbles of vapor in the funnel stem. Connect the condenser and heat slowly. Adjust the rate of refluxing so that from 25 to 35 drops of ether per minute drop from the bottom of the sample tube, taking care to place the latter so that the ether flows down its outer surface instead of down the wall of the extractor jacket. Continue the extraction for 4 hours. When the extraction is complete, remove the sample tube from the jacket by means of a wire hook inserted in the overflow hole and place the jacket and contents to a depth of about 5 cm. in a water bath at 50–60°. Keep the tube in the bath about 15 minutes after all visible ether has evaporated and then transfer it to a 100° bath for 5 minutes. Remove the jacket from the bath and add 0.03 N HCl equal in volume to the original sample which was extracted. (See "Standard concentration curves" above.) Return the jacket to the 55° bath for 10 minutes with gentle mixing and then to the 100° bath for 5 minutes. It has been shown that these heating conditions cause complete solution of the quinine with negligible evaporation of the solvent. Stopper and cool the jacket to room temperature. Rotate the jacket so that the condensate on the inner wall is included with the contents and remove any turbidity (due to extracted lipids) by filtration through a 2.5 cm. funnel, the stem of which is so packed with glass wool that the filtration rate is not over 1 ml. per minute. (Use Pyrex brand fiber glass No. 719.) Use this filtrate directly or store, with proper light protection (see "Quinine standards" above), for the determination of quinine by either the macro- or microprocedure described below.

*Macroprocedure*—Pipette 10 ml. samples (in triplicate) of the unknown in 0.03 N HCl solution into test-tubes selected for use in the Evelyn colorimeter and previously cleaned with chromic acid, rinsed with water and alcohol, and dried in an oven at 110°. Using the 400 m $\mu$  filter, place each tube in the instrument and adjust the resistances to give a galvanometer reading of 100, with subsequent checking of the center setting (see "Notes on operation" of the Rubicon Company). Remove each tube, add 0.2 ml. of 10 per cent silicotungstic acid, and heat the tube by immersion to the depth of the sample in a boiling water bath for 5 minutes. Remove the tube from the boiling water bath and place in a cooling bath of running tap water of from 18–22° for 5 minutes. Allow the tube to stand at room temperature for 5 minutes and then read it in the photoelectric colorimeter and determine the concentration of quinine by means of a previously prepared curve of the galvanometer reading *versus* the quinine concentration. This curve should be prepared as described above with standard quinine solutions.

*Microprocedure*—Pipette 1.25 ml. samples (in triplicate) of the unknown in 0.03 N HCl solution into 3 ml. test-tubes and add, by means of a 0.2 ml. Kahn pipette graduated to 0.001 ml., 0.025 ml. of 10 per cent silicotungstic acid. Suspend the tubes in a boiling water bath for exactly 2 minutes and then in a running water bath at 18–22° for 5 minutes. Pour the suspension of quinine silicotungstate into a clean, dry plunger type of micro cell, taking care to cover the plunger end. Use a previously adjusted blank setting of 25 (see above) and a 400 m $\mu$  filter. Multiply the reading by 4 and determine the quinine content on a previously prepared standard curve as described above.

In practice the levels of concentration of quinine encountered in blood fall within the limits of the curve in Fig. 5; urinary concentrations are, however, subject to much greater variation. For this reason solutions of quinine obtained from urine extractions should be tested by removing a small aliquot and adding in the cold the same ratio of reagent as directed above. The formation of a barely perceptible turbidity in the cold indicates a 1:5 dilution to be made; whereas a definite turbidity in the cold indicates a 1:10 dilution. By this means the production of a readable turbidity is assured.

## SUMMARY

A detailed study has been made of the method of determining small amounts of quinine in blood and other biological fluids by precipitation as the silicotungstate.

The nephelometric method for quinine silicotungstate has been adapted to use in the Evelyn photoelectric colorimeter and satisfactory procedures are described for both macro- and micro-determinations.

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## THE ACTIVATION OF INTRACELLULAR PROTEINASES

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Preparations of intracellular proteinases, as obtained from plant or animal material, usually contain natural activators. In the present communication it is shown that the response of intracellular proteinases to added activators may be different, depending upon the presence or absence of natural activators. Even minute quantities of natural activators may influence decisively the course of the activation. For example, it has been found that the most widely studied activation phenomenon, the activation of papain by HCN, is due to the presence of traces of natural activators and that the removal of these natural activators through careful dialysis renders the papain unactivatable by HCN.

*Activation of Papain by HCN*—A solution of papain was thoroughly dialyzed, as described in the experimental section. A solution of this dialyzed papain was found to be inactive toward benzoyl-*l*-arginineamide when no activator was added. After addition of HCN, no increase in enzymatic activity was observed. However, when a very small quantity of H<sub>2</sub>S, cysteine, or glutathione was added to the dialyzed enzyme solution together with the HCN, a high activity toward benzoylarginineamide resulted. These small quantities of sulfhydryl compounds, when employed in the absence of HCN, were insufficient to cause any activation of the papain (Table I).

Furthermore, the activation of the dialyzed papain by HCN in the presence of traces of cysteine was found to be reversed when the HCN was removed *in vacuo*. After evacuation, the enzyme solution was inactive toward benzoylarginineamide. Addition of fresh HCN restored the activity (Table II).

The fact that dialysis removes from papain a substance essential for HCN activation of the papain is shown by the following experiment. The dialysate obtained in preparing the dialyzed papain was concentrated to a small volume. When this concentrated dialysate and the dialyzed papain were mixed in the original proportions and the mixture was treated with HCN, the

TABLE I

*HCN-Activation of Papain. Effect of Traces of Sulfhydryl Compounds*

Substrate, benzoyl-L-arginineamide; enzyme, dialyzed papain, 0.0166 mg. of protein N per cc. of test solution. Temperature, 40°; pH 5.1 to 5.3.

Activator	Activator concentration in test solution	$K \times 10^4$ *
	<i>mM per cc.</i>	
None. ....	0	0†
HCN .....	0.020	0-5
Cysteine .. .	0.00004	0†
{ Cysteine. ....	0.00004	20
{ HCN .....	0.020	
H <sub>2</sub> S .....	0.00004	0
{ H <sub>2</sub> S... ..	0.00004	19
{ HCN.....	0.020	
Glutathione... ..	0.00004	0†
{ Glutathione....	0.00004	31
{ HCN.....	0.020	
Cysteine. ....	0.020	33
H <sub>2</sub> S.....	0.016	12
Glutathione... ..	0.020	30

\* $K = 1/t \log (a)/(a-x)$ . Each value of  $K$  in this and in the following tables represents the average of several constants calculated, as previously described (1).

† These values were determined by carrying out all operations in the complete absence of oxygen to prevent oxidation of the trace of sulfhydryl compound added. In the remaining experiments, no precautions were taken to avoid oxidation. Consequently, the possibility exists that the effective concentration of the sulfhydryl compounds in the latter experiments might be even less than that indicated above.

resulting enzyme solution was highly active. The subsequent removal of HCN *in vacuo* resulted in an inactivation (Table II).

As was to be expected from the preceding experiment, a sample of the original undialyzed papain became highly active on addition of HCN and lost its activity on subsequent removal of the HCN *in vacuo* (Table II).

From these experiments it must be concluded that papain<sup>1</sup> exists in two forms: one of these ( $\alpha$ -papain) is not activatable by HCN but may be transformed by SH compounds into another form ( $\beta$ -papain) that is activatable by HCN. The fact that this

TABLE II

*HCN-Activation of Papain. Effect of Removal of HCN in Vacuo*  
Substrate, benzoyl-L-arginineamide. Temperature, 40°; pH 5.1 to 5.3.

Enzyme preparation	Enzyme concentration in test solution	Treatment*	$K \times 10^4$
	<i>mg. protein N per cc.</i>		
Dialyzed papain	0.0166	(a) None	0
	0.0166	(b) Cysteine (0.00004) + HCN (0.020)	32
	0.0166	(c) (b) after evacuation	0
	0.0166	(d) (c) + HCN (0.025)	31
	0.0166	(e) Cysteine (0.020)	33
Dialyzed papain + dialysate†	0.0432	(a) None	0
	0.0155	(b) HCN (0.020)	17
	0.0155	(c) (b) after evacuation	3
	0.0155	(d) (c) + HCN (0.025)	16
	0.0155	(e) Cysteine (0.020)	35
Undialyzed papain‡	0.0165	(a) None	0
	0.0165	(b) HCN (0.020)	18
	0.0165	(c) (b) after evacuation	3
	0.0165	(d) (c) + HCN (0.025)	17
	0.0165	(e) Cysteine (0.020)	36
Dialysate from papain	0	(a) HCN (0.020)	0
	0	(b) (a) after evacuation	0
	0.0171	(c) (b) + dialyzed papain§	0

\* The figures in parentheses denote activator concentration in mm per cc. of test solution.

† Dialyzed enzyme and dialysate were mixed in the proportions originally present in the undialyzed enzyme.

‡ The procedure employed in this experiment has been described in detail in the experimental section.

§ The concentration of the treated dialysate used was equivalent to 4 times that originally present in the undialyzed papain.

<sup>1</sup> Since papain probably represents a mixture of enzymes, the conclusions drawn in this paper are valid only for the component of HCN-papain that hydrolyzes benzoylarginineamide. It is probable that other components of papain are activated through the formation of similar compounds with HCN.

activation by HCN may be reversed under conditions that exclude oxidation by atmospheric oxygen indicates that the HCN activation consists in the formation of a dissociable HCN- $\beta$ -papain compound.

The activation of undialyzed papain by HCN is due to the presence of natural activators; it must be assumed that a small quantity of these natural activators is present in the SH form and that this trace acts in the same manner as shown above for minute amounts of  $H_2S$ , cysteine, or glutathione.

It is obvious from the foregoing that if, on addition of HCN, a papain preparation acquires the ability to hydrolyze benzoyl-arginineamide it may be concluded that the papain preparation must contain at least a small quantity of a natural activator.

In a previous communication (2) it has been reported that papain becomes inactive when precipitated by means of isopropyl alcohol from a solution of HCN-activated papain. In the opinion of Greenberg and Winnick (3), this result is due to mild oxidation of the active papain by oxygen and not to the dissociation of an enzyme-activator compound. It is clear from the above experiments on the inactivation of HCN-papain by removal of HCN *in vacuo* that this conclusion of Greenberg and Winnick must be regarded as erroneous. Furthermore, in repeating the above experiment, Greenberg and Winnick activated the papain before precipitation, not with HCN, but with cysteine. It might be expected that under these conditions a sufficient quantity of cysteine would be precipitated with the papain to activate the redissolved papain.

*Activation of Papain by  $H_2S$* —A solution of dialyzed papain was inactive toward benzoylarginineamide but became active toward this substrate after treatment with  $H_2S$ . When the enzyme solution was freed of  $H_2S$  by evacuation, the resulting solution became nearly inactive toward the substrate. Addition of fresh  $H_2S$  restored the activity completely (Table III).

However, when the undialyzed papain was activated by  $H_2S$ , the subsequent removal of  $H_2S$  *in vacuo* caused no loss in activity. Identical results were obtained when the same procedure was applied to a mixture of the dialyzed papain and its dialysate (Table III). The difference in the behavior of dialyzed and undialyzed papain toward  $H_2S$  is apparently due to the fact that

undialyzed papain contains substances that are transformed into activators by  $H_2S$ . These substances are removed on dialysis. This conclusion was verified as follows: The concentrated dialysate was treated with  $H_2S$  and the  $H_2S$  was then removed by evacuation. Following this procedure the treated dialysate was added

TABLE III

*H<sub>2</sub>S-Activation of Papain. Effect of Removal of H<sub>2</sub>S in Vacuo*  
Substrate, benzoyl-L-arginineamide. Temperature, 40°; pH 5.1 to 5.3.

Enzyme preparation	Enzyme concentration in test solution	Treatment*	$K \times 10^4$
	<i>mg. protein N per cc.</i>		
Dialyzed papain	0.0302	(a) None	1
	0.0169	(b) $H_2S$ (0.016)	19
	0.0169	(c) (b) after evacuation	3
	0.0169	(d) (c) + $H_2S$ (0.016)	18
Undialyzed papain	0.0165	(a) None	0
	0.0165	(b) $H_2S$ (0.016)	17
	0.0165	(c) (b) after evacuation	16
	0.0165	(d) (c) + $H_2S$ (0.016)	15
Dialyzed papain + dialysate†	0.0302	(a) None	0
	0.0169	(b) $H_2S$ (0.016)	14
	0.0169	(c) (b) after evacuation	17
Dialysate from papain	0	(a) $H_2S$ (0.016)	0
	0	(b) (a) after evacuation	0
	0.0169	(c) (b) + dialyzed papain‡	8-10

\* The figures in parentheses denote activator concentration in mm per cc. of test solution.

† Dialyzed enzyme and dialysate were mixed in the proportions originally present in the undialyzed enzyme.

‡ The concentration of the treated dialysate used was equivalent to 4 times that originally present in the undialyzed papain. The activator formed by  $H_2S$  treatment of the dialysate is very rapidly destroyed on exposure to air.

to dialyzed papain and the activity of the mixture was determined. The high activity observed indicates that the dialysate contained a substance (or substances) that was transformed into an activator by  $H_2S$  (Table III).

Several years ago, Hellerman and Perkins (4) reported an experiment in which the activity of an  $H_2S$ -activated papain prepa-

ration was not changed by the removal of the  $\text{H}_2\text{S}$  in a stream of nitrogen. In view of the above experiments, it would appear that the papain preparation employed by these workers contained a potential activator.

From the experiments described in this section, it is apparent that the action of  $\text{H}_2\text{S}$  on dialyzed papain consists of two distinct steps. The first involves the transformation of  $\alpha$ -papain into  $\beta$ -papain; the second, the activation of  $\beta$ -papain through the formation of an  $\text{H}_2\text{S}$ - $\beta$ -papain compound. As mentioned in a previous paragraph, the amount of  $\text{H}_2\text{S}$  required to perform the first step is relatively small. However, a much larger amount of  $\text{H}_2\text{S}$  is required to convert  $\beta$ -papain completely into an active enzyme. From the reversible nature of the second step it must be concluded that the active enzyme represents a dissociable  $\text{H}_2\text{S}$ - $\beta$ -papain compound. It will be recalled that  $\text{HCN}$  is not able to transform  $\alpha$ -papain into  $\beta$ -papain, but can combine with  $\beta$ -papain to give an active enzyme. It may be postulated that sulfhydryl compounds such as cysteine or glutathione are, like  $\text{H}_2\text{S}$ , capable of transforming  $\alpha$ -papain into  $\beta$ -papain and also of combining with  $\beta$ -papain to give an active enzyme.

When, however, potential activators are present in a papain solution,  $\text{H}_2\text{S}$  may perform an additional function in the activation process. As was shown above,  $\text{H}_2\text{S}$  may transform the potential natural activator into a product that in turn activates papain. This newly formed activator is not removed on evacuation. Therefore, when the activation of an intracellular enzyme by  $\text{H}_2\text{S}$  is found to be irreversible under the experimental conditions described in this communication, it may be concluded that the enzyme preparation contains a potential activator that is transformed by  $\text{H}_2\text{S}$  into a true activator.

*Nature of the Dialyzable Potential Activator in Papain*—When the concentrated dialysate obtained from papain was treated with  $\text{HCN}$  and the  $\text{HCN}$  was removed *in vacuo*, the resulting solution failed to activate dialyzed papain (Table II). This indicates that the potential activator present in the dialysate is not transformed into a true activator by  $\text{HCN}$ . Apparently the chemical nature of the dialyzable potential activator present in crude papain preparations is such that it is transformed into a true activator by  $\text{H}_2\text{S}$  but not by  $\text{HCN}$ .

Numerous investigators, in particular Grassmann (5) and

Maschmann and Helmert (6), have discussed the presence of disulfide and sulfhydryl compounds in crude papain and their possible rôle as natural activators of the enzyme. Grassmann (7) assumed that HCN activates papain only indirectly. He suggested that there is present in papain a substance that is transformed by HCN into a sulfhydryl compound which in turn activates papain. As experimental support for his view, Grassmann mentioned his observation that the disulfide form of glutathione is reduced to the sulfhydryl form by means of KCN.

It seems necessary to emphasize, however, that the reduction of SS-glutathione by cyanide has been performed only at rather alkaline pH values, while the HCN activation of papain takes place at pH 5. When the disulfide form of glutathione is treated with HCN at pH 5 and the HCN is then removed *in vacuo* with exclusion of oxygen, the resulting solution does not activate dialyzed papain for the hydrolysis of benzoylarginineamide. In other words, HCN does not produce from SS-glutathione at pH 5 a quantity of SH-glutathione sufficient for the activation of papain (Table IV).

When SS-glutathione is treated with  $H_2S$  at pH 5, and the  $H_2S$  is then removed by evacuation, the resulting solution is capable of activating papain for the hydrolysis of benzoylarginineamide (Table IV). It may be concluded, therefore, that, in contrast to the action of HCN,  $H_2S$  forms sufficient SH-glutathione to activate papain. Thus SS-glutathione and the dialyzable potential activator from papain resemble one another in their behavior toward HCN and  $H_2S$  at pH 5. It appears possible that the potential activator removed on dialysis of papain is a disulfide.

*Activation of Cathepsin by HCN*—Beef spleen cathepsin contains an enzymatic component which, on activation by HCN, splits benzoylarginineamide. The following experiments show that the activation mechanism for this component is similar to that demonstrated above for the HCN activation of papain. The activated cathepsin component may be designated "HCN-cathepsin (benzoylarginineamide)." Although it is probable that this cathepsin component is identical with Cathepsin II,<sup>2</sup> the above

<sup>2</sup> Cathepsin II is the enzymatic component of beef spleen extracts that hydrolyzes benzoylarginineamide when cysteine has been added as activator (8).



designation should be employed until experimental proof for the identity has been secured.

A carefully dialyzed solution of beef spleen cathepsin was inactive toward benzoyl-L-arginineamide. Furthermore, on addition of HCN only a slight activity resulted. When a very small quantity of cysteine was added together with HCN, a rapid

TABLE IV  
*Action of HCN and of H<sub>2</sub>S on SS-Glutathione at pH 5*

Substrate, benzoyl-L-arginineamide; enzyme, dialyzed papain, 0.0171 mg. of protein N per cc. of test solution. Temperature, 40°; pH of test solutions 5.1 to 5.3.

The reagents were prepared in the absence of the enzyme and substrate; the enzyme and substrate were then added and the values of  $K_{BAA}$  were determined.

Reagent*	pH of reagent solution	$K \times 10^4$
(a) SS-Glutathione (0.020).....		0
(b) " (0.005) + HCN (0.020)†.....	5.13	14‡
(c) (b) after evacuation.....	5.27	0
(d) (c) + HCN (0.025)†.....	5.24	16‡
(e) SS-Glutathione (0.005) + H <sub>2</sub> S (0.020)†. . .	5.21	13
(f) (e) after evacuation . . . . .	5.32	23
(g) (f) + H <sub>2</sub> S (0.020)†.....	5.30	10
(h) SH-Glutathione (0.010).....		18
(i) " (0.005).....		14

\* The figures in parentheses denote concentrations in mm per cc. of test solution.

† Incubated for 2 hours at 40° before addition of enzyme and substrate. In the case of (e), precipitated sulfur was removed from the incubated solution by centrifugation before tests were performed.

‡ The activation obtained in these cases is due to the presence of a trace of sulfhydryl in the oxidized glutathione. The amount of sulfhydryl present is less than 0.00004 mm per cc. of test solution, as is indicated by the results given in Table I.

hydrolysis of the substrate was observed. This small quantity of cysteine, when employed in the absence of HCN, was insufficient to cause activation of beef spleen cathepsin (Table V). These results indicate that a small amount of cysteine suffices to transform this cathepsin component from an  $\alpha$  form (unactivatable by HCN) into a  $\beta$  form (activatable by HCN).

It was found that this  $\beta$  form is inactivated by removal of HCN *in vacuo*. Addition of fresh HCN to the inactive solution restores the enzymatic activity toward benzoylarginineamide (Table V).

*Reduction Theory of Activation of Intracellular Proteinases*—The activation of the intracellular proteinases has been regarded as a reduction process. The activatable enzymes were believed to be reversibly reducible and oxidizable and to be hydrolytically active only in the reduced state. The reduction and oxidation were supposed to represent the appearance and disappearance of a sulfhydryl group in the enzyme (9). This theory rests upon the experimental finding that active papain solutions may be in-

TABLE V  
*Activation of Beef Spleen Cathepsin*

Substrate, benzoyl-L-arginineamide; enzyme, dialyzed cathepsin, 0.15 mg. of protein N per cc. of test solution. Temperature, 40°; pH 4.7.

Treatment*	$K \times 10^4$
(a) None.....	0
(b) Cysteine (0.020).....	17
(c) " (0.00004).....	0
(d) HCN (0.020).....	2
(e) Cysteine (0.00004) + HCN (0.020).....	14
(f) (e) after evacuation.....	0
(g) (f) + HCN (0.025).....	14

\* The figures in parentheses denote activator concentration in mm per cc. of test solution.

activated by oxidizing agents and may be reactivated by reducing agents (Bersin (10), Hellerman (11), and Purr (12)). However, in these experiments the enzyme preparations had not been completely freed of accompanying natural activators and it was not demonstrated whether the oxidation-reduction procedures employed had oxidized or reduced the enzyme itself or the accompanying natural activators. On the other hand, it has been shown above that the activation of papain and of cathepsin by HCN is reversible under conditions in which oxidation and reduction do not occur. Consequently, when an activatable enzyme of the papain-cathepsin group is inactivated by oxidation procedures and reactivated by reduction procedures, it may be concluded

that the observed effects are due to the oxidation or reduction of accompanying activators.

It was previously shown that the transformation of papain into an active enzyme involves two distinct steps: (1) the conversion of inactive  $\alpha$ -papain into inactive  $\beta$ -papain, and (2) the activation process itself, consisting of the formation of an activator- $\beta$ -papain compound. It should be emphasized that the preceding discussion of the activation process applies only to the second step. It has not been determined whether or not oxidation-reduction processes are involved in the first step.

#### EXPERIMENTAL

##### *Preparation of Enzymes*

*Preparation of "Undialyzed" Papain*—A good grade of vacuum-dried papaya latex was purified by a method similar to that described by Grassmann (5). 250 gm. of finely ground latex were shaken for 1 hour with 2.5 liters of distilled water. The suspension was cooled to 4° and was filtered through a layer of Filter Cel. The filtrate was immersed in an ice bath and  $H_2S$  was passed rapidly through the solution for 6 hours. The solution was filtered rapidly with the aid of Filter Cel and to the clear filtrate (2620 cc.) was added sufficient 95 per cent methanol (7340 cc.) to make the alcohol concentration 70 per cent. The container was tightly stoppered to prevent loss of  $H_2S$  and the precipitated material was allowed to settle overnight in the cold. The precipitate was collected by filtration on a layer of Filter Cel and was washed three times with 70 per cent methanol by suspension and filtration. The washed precipitate was dissolved in water, filtered to remove the Filter Cel, and the filtrate (1160 cc.) was again treated with  $H_2S$  by the above procedure for 4 hours. Methanol (3080 cc.) was added to the clear filtrate (1100 cc.) to make the alcohol concentration 70 per cent, and the precipitate was collected by centrifugation after settling overnight in the cold. The precipitate was washed in closed centrifuge bottles twice with 70 per cent methanol, twice with absolute methanol, and once with ether. The ether was removed by repeated evacuation in the cold and the material was finally dried over  $P_2O_5$  in a vacuum desiccator at room temperature. Yield, 83 gm. Nitrogen, 12.7 per cent; protein nitrogen, 8.3 per cent.

*Preparation of "Dialyzed" Papain*—200 mg. of the above papain preparation were dialyzed against distilled water at 4° for 7 days in a Kunitz dialyzer. The dialyzed enzyme was finally diluted with 12.5 cc. of citrate buffer (0.2 M, pH 5) and distilled water to 50 cc. (0.336 mg. of protein N per cc., 0.336 mg. of total N per cc.). The dialyzed enzyme solution was carefully sealed and stored in a desiccator containing lead acetate solution to prevent contamination by traces of hydrogen sulfide. The dialysate obtained in the above procedure was concentrated *in vacuo* (0.32 mg. of N per cc.; pH of solution, 6.8).

*Preparation of Dialyzed Cathepsin*—Beef spleen cathepsin was prepared as described in a previous communication (13) and was dialyzed at 4° against distilled water for 7 days. The resulting solution contained 1.08 mg. of protein N per cc.

### Methods

The substrate, benzoyl-*L*-arginineamide hydrochloride, was prepared as previously described (14). The rate of hydrolysis was determined by the method of Grassmann and Heyde (15). Each test was performed as follows: 1.0 cc. of an aqueous solution of benzoylarginineamide hydrochloride (41.5 mg. per cc.) and 0.25 cc. of 0.2 M citrate buffer (pH 5.0) were warmed to 40° in a 2.5 cc. volumetric flask. The desired volumes of enzyme and activator solutions were then added, the mixture was diluted to 2.5 cc. with water at 40°, and the incubation was begun. In experiments in which H<sub>2</sub>S or HCN was used, the enzyme, activator, and buffer were first incubated for 2 hours at 40° before addition of the substrate. However, in experiments in which cysteine or glutathione alone was used as the activator, the enzyme, activator, buffer, and substrate were mixed as described above, and measurement of the rate of hydrolysis was begun immediately.

Activator solutions were freshly prepared immediately before use. Cysteine hydrochloride and glutathione solutions were prepared by dissolving the analytically pure compounds in distilled water with sufficient N NaOH to give a pH of 5.0. A solution of HCN at pH 5.0 was prepared by adding the proper amount of N HCl to an ice-cold solution of KCN. A saturated solution of H<sub>2</sub>S (approximately 0.2 M) was prepared by passing the gas into ice-cold, oxygen-free, distilled water. The disulfide form of

glutathione was prepared by treating an ice-cold, neutral solution of pure glutathione with 1 equivalent of hydrogen peroxide. The reaction mixture was shaken for a few seconds and was then evaporated to dryness. The residue was dissolved in water and the resulting solution was adjusted to pH 5.0. Tests for sulfhydryl and peroxide in this solution were negative.

The apparatus used for the removal *in vacuo* of volatile activators from enzyme solutions is shown in Fig. 1.

Nitrogen was purified by passing the gas over hot, reduced copper gauze. The apparatus from *A* to *D* was freed of oxygen by repeatedly evacuating the system and refilling it with nitrogen. During evacuation the opening at *B* was closed and the 2-way stop-cock at *A* was turned to permit the escape of nitrogen through the

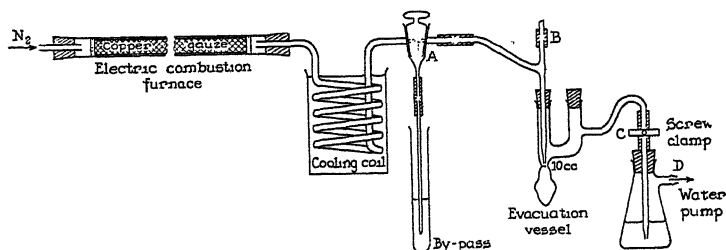


FIG. 1. Apparatus for evacuation of enzyme solutions in an atmosphere of nitrogen. *A*, 2-way stop-cock; *B*, inlet tube; *C*, screw-clamp.

by-pass. After evacuation the clamp at *C* was closed and nitrogen was admitted to the system by turning the stop-cock *A*. Nitrogen was then allowed to escape through the opening at *B* for a few minutes, *C* was opened, and the test solution (usually 5.0 cc.) was introduced through *B* by means of a pipette. The solution was washed into the vessel by means of oxygen-free distilled water (2.0 cc.), a drop of octyl alcohol was added to reduce foaming, and the opening at *B* was closed. The stop-cock at *A* was turned to the by-pass and the system was carefully evacuated to remove the volatile activator. The trap flask contained 20 per cent lead acetate solution in the  $H_2S$  experiments and 0.5 *N* silver nitrate solution in the  $HCN$  experiments. After most of the dissolved gas had been removed, the evacuation vessel was immersed in a water bath and the temperature was raised to 35–40°.

Evacuation at approximately 15 mm. was continued for  $1\frac{1}{2}$  hours at this temperature. At the end of this period the system was filled with nitrogen, as described above, and oxygen-free water was added through *B* to dilute the small residue (approximately 0.5 cc.) to the 10.0 cc. mark. The vessel was removed from the apparatus, stoppered tightly, and the contents were mixed. Portions of this solution were then tested for enzymatic activity and treated as indicated in Tables II to V. Test experiments were carried out with papain, activated by such amounts of cysteine that oxidation would have resulted in a decrease of the velocity constant. Under the conditions described above, no decrease in the velocity constant was observed.

A detailed description of the procedure followed in carrying out a typical evacuation experiment (undialyzed papain, Table II) is given below. The numbers used to designate the steps in the procedure correspond to those given in Table II.

50 mg. of undialyzed papain (4.126 mg. of protein nitrogen) were dissolved in 6.25 cc. of 0.2 M citrate buffer, pH 5.0, and enough distilled water to make the volume 25.0 cc. The activity of this solution (*a*) toward benzoyl-*L*-arginineamide was determined as previously described, 0.25 cc. of the enzyme being used in the 2.5 cc. test flask. The activity of the enzyme in the presence of an excess of cysteine (*e*) was determined in the same manner on another 0.25 cc. portion of the enzyme, 0.5 cc. of 0.1 M cysteine solution (78.8 mg. of cysteine hydrochloride + 0.4 cc. of N NaOH in 5.0 cc.) being used as the activator.

12.5 cc. of the untreated papain (Solution *a*) were treated with 10 cc. of 0.25 M HCN solution (407 mg. of KCN dissolved in 15 cc. of distilled water in an ice bath, plus 6.0 cc. of N HCl, diluted to 25.0 cc.). The mixture was diluted to 25.0 cc. with citrate buffer and was incubated for 2 hours at 40°. The velocity constant ( $K_{BAA}$ ) was determined as before, with 0.5 cc. of this enzyme solution (*b*). 5.0 cc. of the HCN-activated papain (*b*) were evacuated and diluted to 10.0 cc. as described above. The  $K_{BAA}$  was determined with 1.0 cc. of this enzyme solution (*c*). 4.0 cc. of solution (*c*) were then treated with 1.0 cc. of 0.25 M HCN and the mixture was incubated for 2 hours at 40°. The reactivated enzyme was again tested on 1.25 cc. of this enzyme solution (*d*).

In all evacuation experiments controls were run which showed

that the change in activity was due to the evacuation and not to other experimental conditions.

#### SUMMARY

The activation of the components of papain and of beef spleen cathepsin that hydrolyze benzoyl-*L*-arginineamide has been studied by means of reaction kinetics. Each of these components has been found to exist in two inactive forms ( $\alpha$  and  $\beta$  form). The  $\alpha$  form is not activated by HCN, but may be converted into the  $\beta$  form which is then activated by HCN. The activation of the  $\beta$  form by HCN or H<sub>2</sub>S consists in the formation of dissociable compounds of the  $\beta$  form with the activator. Furthermore, the activation and inactivation of the  $\beta$  form can be accomplished without the mutual transformation of SS and SH groups and without the occurrence of reduction and oxidation processes.

The natural activators usually present in papain and cathepsin preparations have been found to influence the effect of added activators in several ways. In order to obtain unambiguous information about the effect of added activators, the natural activators must be removed.

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# A STUDY OF THE SUPPOSED CONVERSION OF PROTOPORPHYRIN TO COPROPORPHYRIN BY THE LIVER

## I. THE FATE OF PARENTERALLY ADMINISTERED PROTOPORPHYRIN IN DOGS WITH BILE RENAL FISTULAE\*

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Van den Bergh and his associates (1) described the appearance of coproporphyrin in the bile obtained by perfusion of surviving rabbit livers with blood to which protoporphyrin had been added. The results of these experiments appeared to be so definite that they were widely accepted as proving the ability of liver tissue to convert proto- to coproporphyrin, and as indicating a close relationship between the protoporphyrin of the red blood cells and the coproporphyrin of the bile and urine. Such a relationship was suggested by simultaneous quantitative studies of the urinary coproporphyrin and the erythrocyte protoporphyrin, as reported by van den Bergh and his coworkers (1). Subsequent work revealed, however, that the erythrocyte protoporphyrin (2) resides in the reticulocytes (3), and that it corresponds in configuration to etioporphyrin III (4), while the coproporphyrin of human bile (5) and at least the major fraction of the coproporphyrin of normal human urine belong to isomer Type I (6-8). The general nature and details of this problem have been discussed fully in recent publications (9-11). Suffice it to say that the considerations just mentioned, together with the importance which the question of conversion of proto- to coproporphyrin has for the whole problem of porphyrin metabolism, induced us to carry out the experimental studies described in the following. The first of these was intended

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† Deceased.



to discover whether the parenteral injection of crystalline protoporphyrin in dogs with bile renal fistulae would be followed by an increased excretion of either coproporphyrin or bilirubin. It was also our purpose to determine, if possible, the isomer type of the coproporphyrin, should an increased excretion occur.

### *Methods*

Bile renal fistulae were prepared<sup>1</sup> in two adult mongrel dogs of medium weight, according to the method of Kapsinow and co-workers (12). During the experimental period the animals were maintained on a uniform diet consisting of Pard dog food plus bone ash. 2 gm. of bile salts<sup>2</sup> were given daily. The dogs were kept in metabolism cages permitting separate collection of the combined 24 hour urine-bile samples and the feces, which were collected for consecutive 4 day periods.

The feces urobilinogen was determined by the method of Watson (13). Bilirubin in the combined urine-bile samples was determined by the Jendrassik-Czike modification (14) of the van den Bergh method. 2 cc. of the urine-bile sample were mixed with 1 cc. of the diazo reagent. The latter was prepared as follows: 9.9 cc. of sulfanilic acid solution (5 gm. of sulfanilic acid and 50 cc. of concentrated HCl made up to 1 liter with distilled water) were mixed with 0.1 cc. of 0.5 per cent sodium nitrite solution. 0.5 cc. of a saturated aqueous solution of caffeine sodium benzoate was added after the diazo reagent. This was followed in 5 minutes by 6.7 cc. of 95 per cent alcohol, and finally by 1 cc. of concentrated (37.5 per cent) HCl. The azobilirubin was determined with the Zeiss stufenphotometer by means of Heilmeyer's values (15). These are tabulated in Urbach's monograph (16).

The porphyrins in the urine-bile samples were fractionated and determined as follows: 50 cc. of the sample were acidified strongly with glacial acetic acid and extracted in a separatory funnel with 20 to 30 cc. of ether. This extraction was repeated twice. The combined ether extract was washed out twice with distilled water and then extracted repeatedly with small amounts (2 to 4 cc. portions) of dilute HCl until the last extract exhibited either

<sup>1</sup> We are indebted to Dr. Louis Sperling of the Department of Surgery for carrying out these operations.

<sup>2</sup> Bilon, supplied through the courtesy of Eli Lilly and Company.

very faint or no red fluorescence in Wood's light. The source of the latter was an ordinary carbon arc with a Corning red-purple ultrafilter No. 587. In the first experiment (Table I, Period 1) the total porphyrin was extracted from the ether with 5 per cent HCl. Protoporphyrin was then removed from the 5 per cent HCl by repeated extraction with chloroform, the coproporphyrin remaining in the HCl. The chloroform was mixed with several volumes of ether, after which the protoporphyrin was removed with 5 per cent HCl. In all subsequent experiments, the primary ether was extracted first with 1 per cent HCl (four to six extractions) which removed the coproporphyrin and then with 5 per cent HCl (three to four extractions) to remove the protoporphyrin. The 1 and 5 per cent HCl was prepared by dilution of 10 and 50 cc. respectively of concentrated HCl (37.5 per cent) to 375 cc. with distilled water. The ether-HCl fractionation was repeated once with both copro- and protoporphyrin fractions. The fluorimetric determinations of coproporphyrin were carried out in 1 per cent HCl; those of protoporphyrin in 2 per cent HCl. The intensity of fluorescence was measured by comparison with a standard coproporphyrin (I) solution (1 mg. in 100 cc. of 1 per cent HCl) in a Zeiss stufenphotometer. The light source was a small, high pressure mercury arc lamp (Mico type) firmly attached to the front of the photometer. The light was filtered through a heat-resisting red-purple ultrafilter, Corning No. 587. The standard coproporphyrin solution was also employed in determining the intensity of fluorescence of the final protoporphyrin solutions. Direct comparison of a freshly prepared protoporphyrin solution in 2 per cent HCl with the standard coproporphyrin solution revealed that the intensity of fluorescence was approximately the same. The relative lability of protoporphyrin, especially in ultraviolet light, excluded the possibility of employing a standard protoporphyrin solution.

The protoporphyrin for parenteral administration was prepared from crystalline hemin by the formic acid-iron method of Fischer and Pützer (17). The porphyrin was recrystallized from pyridine-water according to Fischer and Pützer's method. Solutions for injection were prepared by dissolving 50 mg. of the crystals in 1.5 cc. of 10 per cent ammonium hydroxide. This was diluted with distilled water and nearly neutralized with 10 per cent HCl.

TABLE I

*Excretion of Proto- and Coproporphyrin and Bilirubin in Dog 3, with Bile Renal Fistula, during Parenteral Administration of Protoporphyrin*

Period No.	Date	Proto-porphyrin administered	Volume of urine and bile	Protoporphyrin	Coproporphyrin	Bilirubin
	1937-38	mg.	cc.	γ per day	γ per day	mg. per day
1	Dec. 27-Jan. 1 1938		400- 740 (574)	80-208 (142)	35-108 (70)	95.6-125 (92)
	Jan. 1	100*				
	" 2-5		535- 650 (592)	2950-345 (1452)	773-232 (496)	73.9- 80.2 (72.9)
2	Mar. 19-26		650- 730 (690)	84†	41†	92.8-130.3 (106)
	" 26	50*				
	" 27-29		600- 705 (657)	1269‡	243‡	148.5- 93.5 (112)
3	" 30-Apr. 3		640- 720 (696)	45§	148§	98.6-110.1 (106)
	Apr. 3	50				
	" 4	50¶				
	" 4-6		780-1120 (950)	1610**	249**	145.4-118.1 (131.7)
	" 7-14		530- 820 (725)	85- 91††	155-172††	91.5-161.7 (115.4)
4	" 24-May 1		575- 770 (657)	41- 61 (52)	112-184 (139)	107.9-124.4 (114.3)
	May 1	120*				
	" 2-5		505- 665 (598)	1470-282 (876)	612-127 (369)	84.3- 90.3 (86.7)
	" 6-17		415- 780 (595)	166- 84 (121)	179- 95 (132)	68.5-119.5 (90.7)

The figures in parentheses are averages.

\* Intravenously.

† One determination only, March 23.

‡ One determination only, March 27.

§ One determination only, average for March 30 to April 2, inclusive.

|| Intravenously. 6 hours later 50 mg. more were injected, partly intravenously, partly subcutaneously.

¶ Largely subcutaneously and intramuscularly. 6 hours later, 50 mg. intravenously.

\*\* Average *per diem* excretion (one determination).

†† Average *per diem* excretion.

(Prompt precipitation occurs if the solution is made acid to litmus.) The volume was then made up to 20 cc., the amount usually employed for one injection. As noted in Tables I and II, the protoporphyrin was injected intravenously for the most part, at times subcutaneously or intramuscularly.

The isolation and identification of coproporphyrin from certain of the urine-bile samples, as indicated in the following, was carried out in the usual way (5). After as much crystallization as possible was achieved by further concentration of the mother liquors,

TABLE II

*Excretion of Proto- and Coproporphyrins and Bilirubin in Dog 4, with Bile Renal Fistula, before and after Intravenous Administration of Protoporphyrin*

Date	Protoporphyrin administered	Volume of urine and bile	Protoporphyrin	Coproporphyrin	Bilirubin
1938	mg.	cc.	$\gamma$ per day	$\gamma$ per day	mg. per day
Apr. 7-14		520-985 (727) *	14*	116*	52 -107.8 (75.7)
" 14	50†				
" 15		850	1063‡	379‡	77.2
" 15	50				
" 17-18		640-1020 (791)	36-106 (68)	110-136 (121)	45.1-117.3 (72.7)

The figures in parentheses are averages.

\* One determination only, average for April 13 and 14.

† Two doses, 7 hours apart.

‡ Average for April 15 and 16.

the copper complex of the methyl ester was prepared in the usual way (18), and in one instance, as noted in the following, sufficient crystals were obtained from glacial acetic acid for a melting point determination. The Fisher-Johns microapparatus was used for melting point determinations.

### Results

The *per diem* excretion of urobilinogen was usually less than 5 mg., but on a few occasions as high as 10 mg. By contrast with the relatively large amounts of bilirubin in the urine-bile samples,

as shown in Tables I and II, it was clear that most of the bile had been shunted into the renal pelvis, but the persistence of small amounts in the feces, and rarely in the urine (never in excess of 3 mg. daily and usually much less than this), was not explained. Presumably a small fraction of the bile had escaped at the point of fistula and regained access to the lower end of the common duct, even though this was ligated at operation.

The results of the experiments are shown in Tables I and II.

Coproporphyrin isomer Type I was isolated from the combined urine-bile samples of April 3 to 15, Table I; May 1 to 17, Table I; and April 14 to 26, Table II. After repeated recrystallization from chloroform-methyl alcohol, the methyl esters of the first two periods melted sharply at 244–245°, while that from the latter period melted at 241–243°. The crystals of the copper complex obtained from the final mother liquor for the period April 14 to 26 shown in Table II (Dog 4) melted at 234–238° (uncorrected). The copper complex of coproporphyrin I methyl ester melts at 286° according to Fischer and Orth (18), that of coproporphyrin III methyl ester at 177°. In the present instance it was impossible to recrystallize the material isolated because of its very small amount. This may have accounted for the lower melting point noted, although the possibility is not excluded that a small fraction of coproporphyrin III was represented.

#### *Comment*

The only experiment reported in the literature of the type described above was carried out by Vigliani (19) in a patient having a total external biliary fistula. Of the protoporphyrin which was injected intravenously, but a very small fraction was recovered. No increase in coproporphyrin was noted in either urine or bile, although Vigliani found that both coproporphyrins I and III, when injected intravenously in the same patient, reappeared in the bile. In the present experiments, the fate of the major fraction of the protoporphyrin was not ascertained. The data do not support the possibility that the protoporphyrin might have been converted in large part to bilirubin. While it is true that in one instance there appeared to be an increase of bilirubin corresponding to the amount of injected protoporphyrin, this one value (148.5 mg. on March 27, Table I, Period 2) was but slightly larger than values noted when the animal had not received proto-

porphyrin, and conversely, on other occasions immediately after protoporphyrin injections, no increase in bilirubin was observed. While the possibility cannot be wholly excluded that a fraction of the protoporphyrin was converted to bilirubin, this appears unlikely.

The increase of coproporphyrin in the urine-bile samples, following protoporphyrin injection, was slight when compared with the amount of protoporphyrin injected. Furthermore, during the periods of greatest increase, the coproporphyrin excreted was of isomer Type I. This brings up the question of whether a porphyrin of Type III can be converted in the animal organism into a Type I isomer. On theoretical grounds, this question has been answered in the negative ever since Fischer pointed out that, for such a conversion to take place, the porphyrin ring would have to be split to permit rotation of pyrrole nucleus IV by  $180^\circ$ , after which a resynthesis of the molecule would be necessary. This possibility, although it appears unlikely, cannot be excluded at present. Another explanation that might be offered for the considerable increase in coproporphyrin I noted in the above experiments is that the excretion of native coproporphyrin I was for some unknown reason increased. Since the samples studied included the entire bile, the increases cannot be regarded as due to a shunt of coproporphyrin into the urine because of liver dysfunction. The increase must therefore represent an actual increase in formation of coproporphyrin I. The possibility exists that the injected protoporphyrin stimulated erythropoiesis and in this way caused an increased formation of coproporphyrin I such as has been shown to occur in hemolytic jaundice, pernicious anemia (5, 20), and after hemorrhage (21). This seems rather unlikely, however, in view of the fact that no increase of hemoglobin, erythrocytes, or reticulocytes was observed.

It is barely conceivable that the protoporphyrin IX (isomer Type III) which was injected contained very small fractions of a protoporphyrin belonging to isomer Type I. Fischer (22) recently discussed evidence suggesting that samples of mesoporphyrin IX as obtained from human blood might contain some mesoporphyrin II (isomer Type I). In a subsequent publication, however, concrete evidence to the contrary was described by Fischer and Schröder (23).

Further emphasis should be placed upon the marked disparity

between the amount of protoporphyrin injected and the relatively very small amount of coproporphyrin which subsequently appeared in the urine-bile samples. Although the coproporphyrin increased from 3- to 10-fold, the excess over the control periods was never more than one-fiftieth of the injected protoporphyrin, and usually less than this. Such a disparity in itself strongly suggests that the increased coproporphyrin was not derived from the protoporphyrin which was given. Nevertheless, this possibility is not wholly excluded.

It should be noted that in each of the above experiments small amounts of a chloroform-soluble porphyrin of deuterio type appeared in the urine-bile samples after injection of the protoporphyrin. In ether the band of this porphyrin in the red region exhibited maximum absorption at 624  $m\mu$ . The porphyrin was removed from ether by 1 per cent HCl and from 0.2 per cent HCl by chloroform. The absorption maximum in HCl varied on different occasions from 551 to 553  $m\mu$ . It is believed that this porphyrin is a derivative of protoporphyrin; further evidence in support of this view will be mentioned in Paper II of this study.

It should be noted further that the coproporphyrin values shown in the tables are too high due to inclusion of this porphyrin of deuterio type. Undoubtedly it would have been preferable to extract the deuterio fraction with  $\text{CHCl}_3$  from 0.2 per cent rather than 5 per cent HCl. (The 0.2 per cent HCl- $\text{CHCl}_3$  fractionation was employed in the coproporphyrin isolation procedure.) This simply minimizes even more the possibility that the injected protoporphyrin yielded the coproporphyrin I which was isolated.

#### SUMMARY

Studies of the fate of crystalline protoporphyrin injected parenterally in dogs with bile renal fistulae have yielded the following information.

1. The major fraction of the protoporphyrin did not reappear in the urine-bile samples, nor was it represented by any equivalent increase of either bilirubin or coproporphyrin.

2. Determinations of bilirubin excretion failed to indicate that any appreciable fraction of the protoporphyrin was converted to bilirubin.

3. The coproporphyrin excretion in the urine-bile samples increased from 3- to 10-fold. This, however, represented but a minute fraction of the injected protoporphyrin. Furthermore, the increase in coproporphyrin was represented by the Type I isomer, whereas had it been derived from the injected protoporphyrin, much larger amounts of Type III isomer might have been expected. The appearance of a porphyrin of deuterio type was noted after administration of protoporphyrin.

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# A STUDY OF THE SUPPOSED CONVERSION OF PROTOPORPHYRIN TO COPROPORPHYRIN BY THE LIVER

## II. THE PORPHYRIN METABOLISM OF RABBIT LIVER\*

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The results described in Paper I of this study did not provide convincing evidence that the dog liver is capable of converting protoporphyrin into coproporphyrin. On the contrary, they were more nearly in agreement with Vigliani's results (1) for human bile fistula subjects. These results failed to indicate any conversion. The question arose, therefore, as to whether the rabbit liver is peculiarly capable of effecting such a transition (2).

The conclusions of van den Bergh and his coworkers (2) with respect to this conversion by the surviving rabbit liver appeared to be open to doubt both on theoretical and technical grounds. So far as is known, vertebrates lack the enzymatic systems necessary for the direct, step by step, additive building up of carbon chains, such as would be required for a conversion of proto- into coproporphyrin. The identification of the coproporphyrin in van den Bergh's experiments depended solely upon simple spectroscopic methods. We have repeatedly observed, and one of us has previously described (3), a pseudodeuteroporphyrin derived from protoporphyrin and having an absorption spectrum almost indistinguishable from that of coproporphyrin. Because of this, a possibility of confusion obviously existed. With these considerations in mind, van den Bergh's experiments have been repeated and to some degree enlarged upon in the present study.

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## EXPERIMENTAL

*Physiological*—A group of twelve rabbits was treated according to the directions given by van den Bergh *et al.* The animals were killed by exsanguination under local anesthesia. The abdomen was incised in the mid-line and a cannula inserted in the bile duct, the portal vein, and the thoracic portion of the vena cava inferior. The vena cava inferior was then ligated in the abdominal cavity. The liver was perfused with the animal's own defibrinated blood, diluted 1:2 with Tyrode's solution. The perfusion fluid was kept at 37° and 15 to 20 cm. of water pressure. It was oxygenated by air bubbles and passed through a gauze pad before being recirculated. In spite of these precautions the liver gradually became clogged and the volume of circulating fluid became greatly reduced over a period of time. Furthermore, the results of this type of experiment were none too satisfactory because it was impossible to obtain more than 5 cc. of bile in any one of the twelve perfusions. This quantity is not sufficient for adequate characterization of its porphyrin content. The van den Bergh method of liver perfusion might also be considered unphysiological, because the reduced colloid osmotic pressure of the perfusion fluid might be expected to change the enzymatic activity and the membrane characteristics of the liver cells.

To obviate some of the shortcomings of the cruder methods of liver perfusion—like van den Bergh's—it seemed advisable to improve the perfusion method in order to conserve the liver in a more physiological state. In order to have a copious bile secretion of the perfused organ, the flow of oxygenated blood must not be interrupted, the organ must not be subjected to drying, and the temperature regulation must oscillate within a very narrow range. For our experimental purpose a radically new approach was sought, because, even with a complicated perfusion apparatus which might have satisfied our requirements, we could not have collected enough of the animal's own blood to make perfusion possible, and therefore we would have had to use the blood of other animals, thus laying the experiment open to new criticisms.

In order to gain the same information by different methods, we collected the feces of rabbits which had been injected intraportally with protoporphyrin. The animals were kept in metabolism cages and the feces and urine collected for a period of 2 weeks; then they

were anesthetized by intravenous injection of 25 mg. of pentothal sodium per kilo of body weight. The sterile porphyrin preparation was injected into the portal vein through an incision in the abdomen. The abdominal wound was closed and the animal allowed to recover. In most rabbits recovery was uneventful and rapid; at least two out of our series of five animals were eating cabbage within 2 hours after termination of the experiment. The feces of the experimental animals were then collected for a period of 1 week and analyzed for porphyrin content. If protoporphyrin was converted into coproporphyrin, we might have expected to find an increased coproporphyrin content in the feces from the period following the injection, as compared with the control period. The possibility that the bacteria of the rabbit's intestinal tract change the biliary porphyrin is, however, not excluded in this type of experiment and therefore we tried still another experimental approach. Rabbits were subjected to spinal anesthesia according to the method described by Bieter and coworkers (4). This method requires a minimum of anesthetic and it is therefore probable that the enzymatic systems of the liver are not markedly influenced by the anesthesia. The paralyzed and anesthetized rabbits were then washed and shaved and tied to a frame rack. An abdominal incision was made, a cannula inserted in the bile duct, and the animals were then immersed, except their heads, in Ringer's solution which was kept at 37°. Bile was collected after the porphyrin preparation had been injected into the portal vein. The bile was allowed to flow into a stoppered test-tube which was stitched to the animal's peritoneum and which had a second rubber tube leading out into the atmosphere in order to keep the pressure in the collection tube constant. In this type of experiment the liver was in a better condition, as witnessed by the increased biliary flow. The duration of bile collection is limited, however, by the duration of anesthesia and by the onset of toxemia due to bacterial action. During the 1st hour or so of the experiment the rabbits seemed to be in very good condition; some were even eating cabbage when it was offered. The animals soon became drowsy, however, and some of them died after 2 hours or more in the saline bath. We had a number of experiments which were unsuccessful because of the short duration of anesthesia or because the animals suffered from shock or failed to secrete bile.

*Chemical Methods*

The crystalline protoporphyrin used was prepared<sup>1</sup> from hemin according to the method of Fischer and Pützer (5). The crystals were dissolved in saline which had been made slightly alkaline by the addition of ammonia. The pH of the saline did not exceed 9.0. Our protoporphyrin preparation did not dissolve very readily in this solvent; only by prolonged grinding was it possible to obtain a stable suspension. Some material sedimented even from the most stable suspension on standing. Van den Bergh makes no mention of this difficulty in his original paper (2), perhaps because he did not use crystalline material for his experiments and the impure preparations may have dissolved more readily. The precipitation of protoporphyrin from its solution might have been a source of error in the perfusion experiments, and precipitated porphyrin was actually found after the perfusions on the gauze filter. On the other hand, precipitation of porphyrin could not have been very disturbing in the direct injection experiments, because of the gradual dissolution of porphyrin in the body fluids. Excessive amounts of protoporphyrin were not found in the livers of porphyrin-injected animals, another indication that the injected material had been disposed of.

In order to determine the porphyrin content of the various experimental samples, the following procedure was followed: solid samples were ground with sand and extracted with glacial acetic acid-ether in the usual way (6); liquid samples were extracted directly in the manner described for urine (7). All extractions were repeated until no more fluorescence was exhibited. The combined glacial acetic acid-ether extracts were washed repeatedly with small amounts of water and then extracted with 5 per cent HCl, removing the proto-, copro-, and deuteroporphyrins and leaving many of the contaminating substances behind. The 5 per cent HCl solution was then extracted with chloroform to remove protoporphyrin. The chloroform was diluted with several volumes of ether and the protoporphyrin was extracted with 5 per cent HCl. After addition of sodium acetate the porphyrin was again extracted with ether. This last ether was observed for fluorescence and for absorption spectra. The original 5 per

<sup>1</sup> We are indebted to Mr. Samuel Schwartz for preparation of this material.

cent HCl solution was then extracted with ether and the ether extracted with 1.5 per cent HCl. This fraction contains the copro- and the deutero- and pseudodeuteroporphyrins. The 1.5 per cent HCl was diluted to 0.2 per cent HCl and extracted with chloroform. The porphyrins which remained in the 0.2 per cent HCl after chloroform extraction were assumed to be coproporphyrin if they gave the characteristic spectrum and a soluble sodium salt. Porphyrins extracted from 0.2 per cent HCl by chloroform were assumed to belong to the deuteroporphyrin fraction (either deutero- or pseudodeuteroporphyrins). When the amount sufficed, the deutero fraction was further identified by its insolubility in 10 per cent NaOH. In Tables I to IV the mode of identification is indicated in a foot-note. The porphyrin content of the solutions was determined with the Zeiss stufenphotometer as described in Paper I.

### *Results*

Table I shows the results of analysis of the bile obtained after perfusion according to the method of van den Bergh and coworkers. These are control observations in which protoporphyrin was not added to the perfusion fluid. Owing to the small amount of bile produced, the amount of porphyrin which was extracted was too small to lie within the range of accuracy of our method and the figures below 5  $\gamma$  of porphyrin must be interpreted as being not more accurate than  $\pm 50$  per cent. The values for protoporphyrin, and for the deutero fraction, as given in Tables I to IV, are only relative, since they are in terms of the coproporphyrin standard. As noted in Paper I, the values for protoporphyrin are approximately correct by this method, but in the case of the deutero fraction no approximation is possible, owing to lack of knowledge of its exact make-up.

The livers of Rabbits 1 to 3 were perfused without any addition to the perfusion fluid; in the experiments on Rabbits 4 to 7 respectively, 2, 3, 4, and 5 cc. of a 1 per cent solution of ammonia were added to the blood substitute. This addition of ammonia was to serve as a control for the later porphyrin perfusion experiments, because, as stated above, protoporphyrin could be kept in solution only if the latter were weakly ammoniacal.

The *cells* of the perfusion fluid of the experiments on Rabbits

2 to 6 contained traces of protoporphyrin, but no other porphyrins were present.

The non-cellular portion of the perfusion fluid ("serum") of the experiments on Rabbits 1 to 7 contained traces of protoporphyrin, the combined protoporphyrin fractions exhibiting faint absorption

TABLE I

*Porphyrin Excretion in Bile from Rabbit Livers Perfused According to Method of van den Bergh and Coworkers (without Protoporphyrin)*

Rabbit No.	Deutero fraction*	Bile	Perfusion time
	$\gamma$	cc.	hrs.
1	0.7	2.5	3
2	1.5	4.0	3
3	0.7	2.0	2½
4	1.2	2.8	3½
5	1.2	3.5	4
6	1.3	3.7	3
7	1.5	4.3	3½

No coproporphyrin and only traces of protoporphyrin were found.

\* The deutero fraction includes any porphyrins leaving 0.2 per cent HCl for  $\text{CHCl}_3$ .

TABLE II

*Porphyrin Excretion in Bile from Rabbit Livers Perfused with Protoporphyrin (van den Bergh's Method)*

Rabbit No.	Protoporphyrin	Coproporphyrin	Deutero fraction*	Bile	Perfusion time	Protoporphyrin used in perfusion
	$\gamma$	$\gamma$	$\gamma$	cc.	hrs.	mg.
8	2.1	0	Trace	4.5	3½	10
9	0	0	0	0.5	3	15
10	Trace	Trace?	Trace	2.0	3½	12
11	7	0	1.8	2.7	2½	15
12	0.5	Trace	2.0	3.8	3	15

\* The deutero fraction includes any porphyrins leaving 0.2 per cent HCl for  $\text{CHCl}_3$ .

maxima at 600 and 556  $m\mu$  in 5 per cent HCl, and the characteristic band in the red, in ether, having maximum absorption at 632  $m\mu$ . The livers of Rabbits 2 to 6 contained small amounts of protoporphyrin and also traces of the deuteroporphyrin fraction.

Table II contains the results of analysis of experimental material

after perfusion of rabbit livers as in the preceding set of experiments, but with the addition of about 15 mg. of protoporphyrin. The crystalline porphyrin was ground with 15 to 20 cc. of Tyrode's solution which had been made alkaline by the addition of 2 to 5 cc. of 1 per cent ammonia.

The cells of the perfusion fluid contained some protoporphyrin in all five experiments.

The "serum" contained protoporphyrin in all five experiments. In each experiment a small amount of a porphyrin was found which was extracted by 0.5 per cent HCl from ether and from 0.2 per cent HCl by chloroform. In ether this porphyrin exhibited an absorption band in the red region having a maximum at 624  $m\mu$ .

TABLE III

*Fecal Porphyrin Excretion in Rabbits Receiving Intraportal Injections of Protoporphyrin*

Rabbit No.	Feces	Protoporphyrin	Deutero fraction*	Protoporphyrin injected
	<i>gm.</i>	$\gamma$	$\gamma$	<i>mg.</i>
13	105	85	200	15
14	83	119	190	20
15	90	85	314	20
16	110	119	190	20
17	97	73.5	68	12

Coproporphyrin was not present in sufficient quantities to be estimated.

\* Includes any porphyrins leaving 0.2 per cent HCl for  $\text{CHCl}_3$  and yielding an insoluble sodium salt in 10 per cent NaOH.

This absorption, together with the chloroform solubility, indicated the presence of a pseudodeuteroporphyrin. These characteristics, plus the fact that the porphyrin yielded an insoluble sodium salt, clearly excluded coproporphyrin.

The livers contained large amounts of protoporphyrin. The liver of Rabbit 8 contained 32  $\gamma$  and that of Rabbit 11 40  $\gamma$  of the deutero fraction (bands in ether 623.6  $m\mu$ ; in 5 per cent HCl at 550.3  $m\mu$ ).

Table III shows the results of analysis of the feces of rabbits which had been injected intraportally with a solution of protoporphyrin.

The pooled feces of Rabbits 13 to 17 for a period of 14 days



prior to injection of protoporphyrin served as a control for the values shown in Table III. The total weight of the pooled control feces was 950 gm. This amount contained 47  $\gamma$  of protoporphyrin and 146  $\gamma$  of the deutero fraction. There were but traces of coproporphyrin, insufficient to be estimated.

Table IV shows the porphyrin contents of the bile samples secreted by rabbits which were injected with protoporphyrin intraportally and maintained in a saline bath.

TABLE IV  
*Porphyrin Content of Bile after Perfusion of Livers of Surviving Rabbits with Protoporphyrin*

Rabbit No.	Protoporphyrin	Deutero fraction*	Coproporphyrin	Bile	Perfusion time	Protoporphyrin injected
	$\gamma$	$\gamma$	$\gamma$	cc.	hrs.	mg.
18	3.4	4.4	0	10.5	4½	12
19	5.2	3.2	Trace	8.1	4	15
20	4.3	5.1	0	13.2	5	20
21	1.8	3.5	0	7.5	3	15
22	3.6	3.7	Trace	7.9	3½	15

\* Includes any porphyrin leaving 0.2 per cent HCl for  $\text{CHCl}_3$ .

#### DISCUSSION

As a result of the above experiments it seems well established that coproporphyrin does not occur in rabbit bile after perfusion of the liver with protoporphyrin preparations. In only four cases were traces of coproporphyrin observed; and these were too minimal for estimation. It seems probable to us that van den Bergh, Grotepass, and Revers (2) were dealing with a member of the deuteroporphyrin fraction, which they believed to be coproporphyrin. This interpretation is supported by the paper of Fischer and Duesberg (8), who found no coproporphyrin in rabbit feces.

The data shown in Table III make it clear that a marked increase of the deutero fraction occurred in the rabbit feces following intraportal injection of protoporphyrin. As compared with the control period, this was approximately a 12-fold increase. The data in Table IV likewise indicate a significant increase of the biliary deutero fraction after injection of protoporphyrin in the portal vein.

An increased biliary deuterio fraction thus appears to be caused by an increased blood level of protoporphyrin. This fact might be interpreted in one of two ways: either this fraction represents one or more end-products of protoporphyrin metabolism or else it is one of the intermediate products in the main pathway of protoporphyrin metabolism, of which only a small part is excreted while the main quantity is further degraded. The fecal (and biliary) deuterio fraction fails to account for the total quantity of protoporphyrin which has been administered, even if the excreted protoporphyrin is taken into account. Only about 1.5 per cent of the injected protoporphyrin was accounted for by the excretion of proto- and deuteroporphyrins.

It may be emphasized that this is quite similar to the results of the bile renal fistula experiments on dogs described in Paper I. Here the recovery of the injected protoporphyrin was equally small, and there was also an increase in porphyrins of the deuterio type.

#### SUMMARY

1. The report of van den Bergh and coworkers that rabbit liver converts protoporphyrin into coproporphyrin has not been confirmed.

2. After intraportal protoporphyrin injection, rabbits excrete an increased amount of a member of the deuteroporphyrin fraction.

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## THE HEME-GLOBIN LINKAGE OF HEMOGLOBIN

### III. ANALYSIS OF THE CARBON MONOXIDE PRODUCT. THE PANCREATIC DIGESTIONS OF SEVERAL FORMS OF HEMOGLOBIN

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#### *Analysis of the Carbon Monoxide Product*

It was recently reported that CO hemoglobin is relatively more resistant than oxyhemoglobin to digestion by the pancreatic enzymes (1), and that a heme-protein fragment can be precipitated either from the digest solution or from the dialyzed digest by acidification with acetic acid (2). The composition of this CO product has now been investigated.

*Iron Content*—The value of the iron to nitrogen ratio,<sup>1</sup> which in early experiments lay near 0.43, was found to vary, figures of 0.39, 0.33, and even 0.21 occurring with later preparations. This ratio is independent of the method by which the product is isolated from the digest solution. Three samples from the same digest, (a) precipitated by acetic acid after dialysis of the digest for 1 day, (b) precipitated after dialysis for 6 days, and (c) precipitated directly without dialysis and washed several times with water, had values of 0.254, 0.258, and 0.263 respectively. The variation in different lots may be due to a gradual inactivation of the crude pancreatin mixture employed (*cf.* Haurowitz (3)).

A sample of CO product with an iron to nitrogen ratio of 0.432 was found to have an arginine content equivalent to one arginine residue per 10.3 atoms of iron. If the protein fragment of the CO product were a low molecular polypeptide, representing the original heme-protein linkage, with arginine as one of its constituent

<sup>1</sup> This figure represents a ratio by weight.

residues, then a minimum ratio of one arginine residue per 4 atoms of iron would be required.

*Amino Acid Distribution*—Samples of CO product have been analyzed for their amino acid constituents, and the results obtained, along with a typical analysis of hemoglobin, are presented in Table I. Table II presents a comparison of the composi-

TABLE I  
*Nitrogen Distribution in Hemin-Free Hydrolysate from CO Product*

	CO product	CO product	Hemoglobin*
Fe:N value.....	0.238	0.256	0.020
Hydrolysate N taken, gm.....	1.30	0.368	120
Histidine N, %.....	†	13.6	11.8
Arginine " %.....	11.0	14.4	6.0
Lysine N, %.....	4.60	3.12	9.0
Aspartic acid N, %.....	5.51	4.35	4.0
Glutamic acid " %.....	2.36	1.76	2.0
Total N recovered, %.....		37.2	32.8

\* Values for hemoglobin are taken from Bergmann and Niemann (4).

† Lost.

TABLE II  
*Comparison of Nitrogen Distribution of CO Product and Hemin Protease of Haurowitz (3)*

The results are expressed in per cent of hydrolysate nitrogen.

Nitrogen	CO product	Hemin protease
Basic amino acid. ....	31.1	35
Ammonia.....	5.5	3.4
Histidine.....	13.6	14.6
Arginine....	14.4	8.9

tion of our CO product with that of the hemin protease of Haurowitz (3).

The facts that the distribution of nitrogen among the amino acids accounted for is like that of hemoglobin except for the variation in arginine and lysine, and that the total recovered nitrogen is of the same order of magnitude for both the CO product and hemoglobin indicate that the protein fragment is not a low molecular polypeptide. It is evident that the belief expressed earlier re-

garding the character of the CO product is no longer tenable, for any stabilizing influence which carbon monoxide may have on the heme-globin linkage is apparently insufficient to prevent a breakdown of that linkage during pancreatic hydrolysis.

*Pancreatic Digestion of Hemoglobin, Methemoglobin, Oxyhemoglobin, and Carboxyhemoglobin*

The digestion of CO hemoglobin by a crude pancreatin extract has been found to proceed less rapidly than that of oxyhemoglobin (1). In order to determine the behavior of reduced hemoglobin and methemoglobin relative to the oxy and carboxy forms,

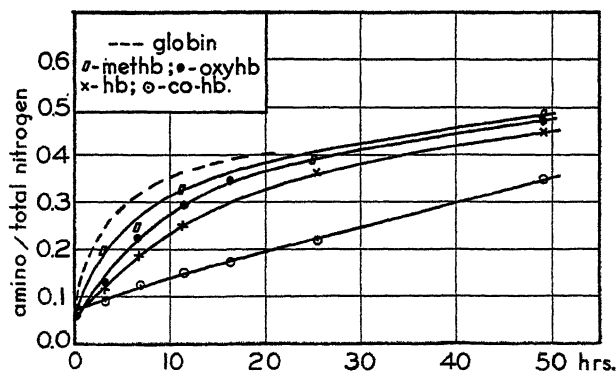


FIG. 1. The digestion of globin, methemoglobin, oxyhemoglobin, hemoglobin, and CO hemoglobin by pancreatin at pH 7.2.

the simultaneous digestion of all four proteins by pancreatin has been investigated.<sup>2</sup>

The data obtained from these studies are charted in Fig. 1, with a similar curve for globin. It is seen that CO hemoglobin is the most resistant to attack by the enzymes, and that reduced hemoglobin approaches it in this respect, being the least rapidly attacked of the remaining forms. At the beginning of the digestion methemoglobin is more quickly split than is oxyhemoglobin; later its rate is about the same as that of oxyhemoglobin. After 7 to 10 days all of the digests approach the same stage,  $0.46 \pm 0.03$  for the amino to total nitrogen ratio.

<sup>2</sup> One of the authors (W. F. R.) wishes to express his indebtedness to Miss Elisabeth L. Johnson for her assistance with the enzyme studies.

Factors other than the condition of the protein substrate do not appear to influence the rates of reaction. The differences between oxy- and CO hemoglobin appear at both acid and basic pH. CO does not retard the splitting of gelatin (1), nor does the presence of CO hemoglobin diminish the rate at which globin is digested by pancreatin; *i.e.*, CO hemoglobin does not inhibit the enzymes. This is shown by the experiment reported in Table III, in which it was found that the increase in amino nitrogen in a mixed digestion of CO hemoglobin and globin was equivalent to the average of the increases in separate digestions of these two substrates. The use of a homogeneous crystalline enzyme, chymotrypsin, instead of the crude pancreatin mixture does not influence the results (Fig. 2).

TABLE III  
*Digestion of Globin by Pancreatin in Presence of CO Hemoglobin*

Time	Ratio, amino to total N			
	COHb (1)	Globin (2)	1:1 mixture of COHb and globin	Arithmetical mean, (1) and (2)
<i>hrs.</i>				
0	0.064	0.070		
6	0.11	0.18	0.16	0.15
19	0.13	0.27	0.19	0.20
24	0.14	0.36	0.22	0.23
71	0.23	0.42	0.28	0.32

The relative behavior of globin and hemoglobin might be expected from the work of Gralén (5), who found that globin has half the molecular weight of hemoglobin. It would therefore offer more surface for union with enzymes than would an equal weight of hemoglobin, thus being more rapidly digested.

If globin were a hemispherical body, two heme groups placed diametrically opposite on the semiplanar surface would enable union between two such halves to give a hemoglobin molecule in which four hemes are located in the central plane of a square. This arrangement would agree with the conclusion reached by Pauling (6) from the oxygen equilibrium data of hemoglobin. The two globin halves might then be held together both by the two coordinate valencies of the heme iron not saturated by por-

pyrin nitrogen (Conant (7)) and by a second kind of linkage, possibly hydrogen bonds, still active when the first type is occupied with a foreign molecule such as oxygen. Reduced hemoglobin having both kinds in force would thus be more resistant to breakdown than oxyhemoglobin. The stability of CO hemoglobin, however, could not be explained in this way.

The dissociation into half molecules undergone by CO hemoglobin in urea (8, 9) would depend upon the rupture of the subsidiary linkages. It would be valuable to determine whether this dissociation is dependent upon the state of the heme in a way corresponding to the digestion rates reported in this paper.

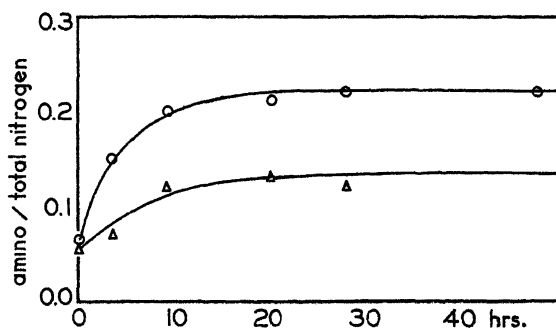


FIG. 2. The digestion of oxyhemoglobin (O) and CO hemoglobin (Δ) by crystalline chymotrypsin at pH 7.5.

#### EXPERIMENTAL

##### *Analysis*

Preparation of the CO product was carried out as previously described (2). However, since it was found that direct precipitation from the digest solution resulted in the same product as that obtained after prolonged dialysis, the rather tedious work of dialyzing large quantities of solution was abandoned, and all products were prepared by precipitation with acetic acid, followed by thorough washing with water containing a few drops of acetic acid. The dry product was used for amino acid analyses. Iron determinations were made by the colorimetric method of Leavell and Ellis (10), total nitrogen analyses by the Kjeldahl method, and amino nitrogen analyses by the volumetric Van Slyke procedure. Amide



nitrogen was determined by hydrolysis of an aliquot of the dry product with 5 per cent HCl for 3 hours, followed by distillation of the liberated ammonia from the hydrolysate at pH 9 into standard acid.

The dicarboxylic acids were first precipitated from the humin-free hydrolysate as their calcium salts by the addition of alcohol and separated by the modified Foreman procedure developed in the laboratory of Chibnall (11). The original mother liquor from the alcohol precipitation and that from the reprecipitation of the calcium salts were combined and analyzed for the basic amino acids by the procedure of Tristram (12).

A sample of dry CO product weighing 5.17 gm. and containing 638 mg. of nitrogen, 163 mg. of iron, 20 mg. of amide nitrogen, and 27 mg. of amino nitrogen was hydrolyzed with 20 per cent HCl for 24 hours. The excess HCl was removed by repeated evaporation in a vacuum, and the humin filtered off and thoroughly washed with hot water. The combined filtrates, representing the total hydrolysate, contained 366 mg. of nitrogen.

The ammonia was removed from this hydrolysate by concentration in a vacuum at pH 8.9 and the dicarboxylic acids precipitated as their calcium salts by the addition of ethyl alcohol. These, after reprecipitation, contained 37 mg. of nitrogen, and 304 mg. of nitrogen remained in the filtrates.

*l-Glutamic Acid*<sup>3</sup>—Glutamic acid hydrochloride was precipitated from the concentrated dicarboxylic acid fraction by saturation with HCl at  $-10^{\circ}$ . The total yield of dry product was 0.085 gm.

$C_5H_9O_4NCl$ . Calculated, N 7.6; found, N 7.8

*l-Aspartic Acid*—The filtrate from the glutamic acid hydrochloride, after removal of HCl, was treated with excess copper carbonate at  $100^{\circ}$ . The copper aspartate thus obtained weighed 0.314 gm. after equilibration with air.

$C_4H_5NCu \cdot 4.5 H_2O$ . Calculated. N 5.07, Cu 23.0  
Found. " 5.04, " 22.4

*l-Histidine*—After the removal of both calcium and chloride ions, histidine was precipitated as its silver salt at pH 7.3. The

<sup>3</sup> Nitrogen analyses on isolated amino acid salts were performed by the Dumas method.

precipitate was decomposed at pH 3.8 by the addition of  $\text{H}_2\text{S}$ , giving a solution containing 83 mg. of nitrogen. 0.927 gm. of histidine diflavianate was obtained from this solution, after concentration to 5 ml., by the addition of 1.25 gm. of flavianic acid.

$(\text{C}_{10}\text{H}_6\text{N}_2\text{SO}_3)_2 \cdot (\text{C}_6\text{H}_3\text{N}_3\text{O}_2)$ . Calculated, N 12.5; found, N 12.4

*l-Arginine*—From the concentrated mother liquor of the silver salt of histidine, arginine silver salt was precipitated at pH 12.5. This on decomposition with  $\text{H}_2\text{S}$  gave a solution containing 66 mg. of nitrogen, to which, after concentration to 8 ml., 0.360 gm. of flavianic acid in 2 ml. of hot water was added. The arginine flavianate after recrystallization weighed 0.458 gm.

$(\text{C}_{10}\text{H}_6\text{N}_2\text{SO}_3) \cdot (\text{C}_6\text{H}_{14}\text{O}_2\text{N}_4)$ . Calculated, N 17.2; found, N 17.0

*l-Lysine*—Excess silver was removed by precipitation as the sulfide in acid medium, the solution concentrated to 30 ml., and lysine phosphotungstate obtained by the addition of 50 ml. of 20 per cent phosphotungstic acid in 5 per cent sulfuric acid. This was decomposed in the usual way, giving a solution containing 27 mg. of nitrogen. This solution was concentrated in the presence of  $\text{BaCO}_3$  to 1 ml., diluted with 6 ml. of alcohol, and treated with 0.230 gm. of picric acid in alcohol at  $0^\circ$ . 0.151 gm. of lysine picrate was obtained.

$(\text{C}_6\text{H}_3\text{O}_7\text{N}_3) \cdot (\text{C}_6\text{H}_{14}\text{O}_2\text{N}_2)$ . Calculated, N 18.7; found, N 18.5

In all cases the mother liquors were worked up for additional product.

*Digestions*—The experimental procedure has already been described (1). For the digestions of Fig. 1 conditions were chosen so that the digest solution contained in 100 ml. 0.5 gm. of substrate nitrogen, 6.0 ml. of pancreatin solution similar to that used earlier, and 20 ml. of borate buffer, pH 8.0, saturated at room temperature. Both substrate and enzyme solutions were brought to  $37^\circ$  before being mixed.

*Methemoglobin*—A solution of oxyhemoglobin containing 27 mg. of nitrogen per ml. was oxidized at pH 8.5 to 9.0, with 1.1 equivalents of potassium ferricyanide. The resulting solution was then dialyzed against running water for 18 hours.

*Hemoglobin*—Oxyhemoglobin in a flask equipped with stop-cocks was freed of oxygen by successive evacuations and flushings

with oxygen-free nitrogen. The digestion was carried out in the same apparatus, care being taken that no oxygen was introduced. The original purple color of reduced hemoglobin was still apparent after a week of digestion.

In the experiments of Fig. 2 chymotrypsin, prepared by the activation of twice recrystallized chymotrypsinogen ((13) p. 136), was employed. The activity of this as well as of the pancreatin solution used above had been determined by the procedure described by Northrop ((13) p. 155). In the digestions of Fig. 2 50 per cent more units of enzyme activity were employed than in those of Fig. 1.

#### SUMMARY

1. The product from the pancreatic digestion of CO hemoglobin has a variable Fe:N ratio, indicating that it is not a chemical unit.
2. The amino acid distribution in the protein part of this product does not support a low molecular polypeptide structure for it.
3. The following order has been found for the rates of digestion of several proteins related to hemoglobin: globin > methemoglobin > oxyhemoglobin > reduced hemoglobin > CO hemoglobin.

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## THE RELATION OF THE VITAMIN B COMPLEX AND LIVER AND PANCREAS EXTRACTS TO FAT SYNTHESIS\*

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The quantitative distribution of fatty acids synthesized in rats by the action of thiamine was reported in a previous communication (1). Characteristic of the fat synthesized under the experimental conditions was the occurrence of a high percentage of  $C_{16}$  acids (41 per cent). The relation of other vitamins of the B complex and of choline to total fat deposition in both the liver and the body of rats has been determined by McHenry and Gavin (2-4).

The nature and quantities of the fatty acids synthesized under some of the above conditions have been determined as a continuation of the earlier work. Interest has centered, in particular, upon the effects of extracts of beef liver and beef pancreas. Confirmation was obtained of McHenry and Gavin's report (4) of the action of a beef liver extract in producing fat synthesis accompanied by an intense fatty liver which was not prevented by choline. The preventive action on the development of such a fatty liver by an extract of beef pancreas, similar to Dragstedt's lipocaic (5) was also confirmed. The action of the liver and pancreatic extracts seems to be related to the metabolism of both glycerides and cholesterol derivatives.

\* A preliminary report of the data on which the present manuscript is based was presented at the meeting of the American Chemical Society at St. Louis, April, 1941. This investigation was aided by a grant from the Buhl Foundation to the University of Pittsburgh and by a grant from the Rockefeller Foundation to the University of Toronto.

## EXPERIMENTAL

Essentially the same procedure described in earlier publications (1-4) was followed in these studies. Young albino rats from the Connaught Laboratories' colony, which had been raised to an average weight of 80 gm. on a stock ration, were placed on a basal ration (1) deficient in the vitamin B complex, free of fat, low in casein, and containing no choline. The ration consisted of cane sugar 84, casein 10, Steenbock-Nelson (6) salt mixture 4, agar-agar 2, and cod liver oil concentrate 0.015. Rats kept on this basal

TABLE I

*Effect of Liver Fraction and Lipocaic on Weight and Lipid Content of Body and Liver*

	No liver fraction		Liver fraction		Liver fraction + lipocaic	
	gm.	gm. per 100 gm. rat	gm.	gm. per 100 gm. rat	gm.	gm. per 100 gm. rat
Average body weight.	70		91		99	
"    liver    "	3.17	4.53	5.42	5.95	4.89	4.94
Carcass lipids, per animal						
Total.....	3.76	5.37	7.83	8.60	9.44	9.53
Acetone-soluble....	3.56	5.09	7.36	8.09	9.34	9.43
Liver lipids, per animal						
Total.....	0.118	(3.71)*	0.650	(12.00)*	0.154	(3.15)*
Acetone-soluble ..	(73.1% lipid)	total	(91.7% lipid)	total	(69.0% lipid)	total

\* Gm. per 100 gm. of liver.

ration for 20 days showed a decrease in average body weight to 58 gm. Following this preliminary period, the animals were continued on the same basal ration and were given subcutaneous injections of B vitamins and choline in the following amounts per rat per day: thiamine hydrochloride (Merck), 12.5  $\gamma$ , riboflavin (Hoffmann-La Roche), 10.0  $\gamma$ , pyridoxine (Merck), 20.0  $\gamma$ , and choline chloride (British Drug Houses), calculated as free base, 10 mg. These supplements only were provided to each of thirty-five animals; a second group of nineteen animals received these supplements and 0.75 cc. of a beef liver extract (4), equivalent to 10 gm. of raw liver; in addition to the B vitamins and choline, a

third group of forty-four animals was given 0.75 cc. of the liver extract and 0.3 gm. of lipocaic (kindly donated by Eli Lilly and Company). The choline equivalent of this amount of pancreas extract was 1 mg., and of the liver extract 15 mg. as determined by the method of Fletcher, Best, and Solandt (7). The duration of the supplementary feeding period was 11 days in all cases. For simplification, these three groups of animals are later referred to as "no liver fraction," "liver fraction," and "liver fraction plus lipocaic." The weights of these three groups of animals are recorded in Table I together with the values obtained for the

TABLE II  
*General Characteristics of Body and Liver Lipids*

Lipids	Saponi- fication equiva- lent	Iodine No. (Wijs)	Lead salt separation of mixed fatty acids				
			Solid acids		Liquid acids		Recov- ery
			gm.	per cent total acids	gm.	per cent total acids	per cent
Carcass glycerides							
No liver fraction	279.5	57.8	34.40	34.8	64.36	65.2	99.1
Liver fraction	281.2	60.9	30.91	26.9	83.80	73.1	99.9
" " + lipocaic	284.3	63.3	25.01	27.6	65.52	72.4	99.3
Liver glycerides							
No liver fraction		72.3	0.43	25.9	1.23	74.1	93.2
Liver fraction		76.7	2.56	29.3	6.17	70.7	96.0
" " + lipocaic		88.2	0.49	16.5	2.47	83.5	94.3

carcass and liver lipids. The composition of the glycerides in the acetone-soluble portion of these lipids was determined by procedures described previously (8). Results of preliminary lead salt separations are given in Table II and in Table III the distillation and analytical data for the methyl ester mixtures are presented. From the latter data, the percentage composition of the original acid mixtures (Table IV) was calculated (*cf.* (9)) for the carcass glycerides. Similar data were obtained and used to determine the composition of the acids of the acetone-soluble liver lipids (Table V). The distribution of lipids in the livers is recorded in Table VI. For the cholesterol analyses, Kelsey's procedure (10) was used.

TABLE III  
*Distillation and Analyses of Methyl Ester Mixtures from Carcass Glycerides*

	Ester fraction	No liver fraction			Liver fraction			Liver fraction + lipocals		
		Weight	Saponi- fication equivalent	Iodine value (Wtgs)	Weight	Saponi- fication equivalent	Iodine value (Wtgs)	Weight	Saponi- fication equivalent	Iodine value (Wtgs)
Liquids	L1	gm. 0.942	243.2	64.7	gm. 2.125	255.6	64.1	gm. 1.384	253.8	67.3
	L2	3.883	255.6	67.8	4.225	263.2	68.1	2.552	269.8	78.5
	L3	3.762	270.2	75.6	5.664	273.9	78.1	4.215	271.5	78.7
	L4	5.033	275.3	83.2	7.010	284.2	79.6	6.070	275.6	80.4
	L5	7.534	282.3	84.0	9.127	286.8	82.1	5.639	277.0	81.3
	L6	13.587	287.4	85.7	8.332	289.3	82.8	9.229	292.6	89.4
	L7	8.305	290.3	87.2	14.169	291.4	83.3	7.768	294.8	90.0
	L8	7.386	292.8	89.4	9.361	293.1	85.6	7.816	295.9	90.1
	L9	4.679	293.2	93.5	7.847	294.8	87.0	2.258	451	94.7
	L10	1.389	295.9	95.2	4.228	295.2	89.0		(302)	(93.2)
	L11	1.938	350	120	3.111	298.0	90.5			
Solids	Total	58.438	(303)	(103)	75.199	(297)	(90.5)	46.931		
	S1	1.312	253.0	1.8	1.677	250.0	1.2	2.257	258.7	1.8
	S2	2.555	263.6	1.9	1.918	262.0	1.0	3.286	266.0	2.0
	S3	4.847	269.3	2.4	6.492	268.2	0.6	7.303	269.9	3.0
	S4	7.621	270.0	3.6	6.491	270.2	1.5	1.961	275.4	3.8
	S5	6.200	271.3	4.3	7.046	270.4	1.5	2.446	281.3	4.6
	S6	5.593	283.0	10.5	2.197	276.2	7.1	2.676	287.2	7.4
	S7	2.021	308	19.2	2.939	294.3	9.7	2.790	306.0	21.0
	S8		(299)		1.619	320.2	17.0		(295.8)	
	Total	30.149			30.379	(296.2)		22.719		

The values in parentheses are for methyl esters after the removal of unsaponifiable material.

TABLE IV  
Fatty Acid Composition of Carcass Glycerides

Acids	Weight percentages			Molar percentages		
	No liver fraction	Liver fraction	Liver fraction + lipocalc	No liver fraction	Liver fraction	Liver fraction + lipocalc
Myristic.....	2.9	2.4	1.9	3.3	2.9	2.3
Palmitic.....	32.5	27.5	23.5	33.8	28.8	24.6
Stearic.....	4.1	3.4	5.7	3.8	3.2	5.4
Arachidic.....	0.1			0.1		
Tetradecenoic.....	1.8	1.0	0.7	2.1	1.2	0.8
Hexadecenoic.....	15.0	13.0	20.3	15.8	13.8	21.4
Oleic.....	39.0	50.1	45.6	36.7	47.6	43.3
Linoleic.....	4.5	2.6	2.3	4.3	2.5	2.2
Arachidonic.....	0.1		Trace	0.1		Trace
Total C <sub>14</sub> .....	4.7	3.4	2.6	5.4	4.1	3.1
“ C <sub>16</sub> .....	47.5	40.5	43.8	49.6	42.6	46.0
“ C <sub>18</sub> .....	47.6	56.1	53.6	44.8	53.3	50.9
“ C <sub>20</sub> .....	0.2			0.2		Trace
Saturated.....	39.6	33.3	31.1	41.0	34.9	32.3
Unsaturated.....	60.4	65.7	68.9	59.0	65.1	67.7

TABLE V  
Composition of Fatty Acids in Acetone-Soluble Liver Lipids

Acids	No liver fraction	Liver fraction	Liver fraction + lipocalc
	molar per cent	molar per cent	molar per cent
Myristic.....	1	1	1
Palmitic.....	32	29	24
Stearic.....	3	3	4
Arachidic.....	3	2	3
Hexadecenoic.....	16	12	18
Oleic ..	39	49	45
Linoleic.....	3	2	3
Arachidonic.....	3	2	2
Total C <sub>14</sub> ..	1	1	1
“ C <sub>16</sub> .....	48	41	42
“ C <sub>18</sub> .....	45	54	52
“ C <sub>20</sub> .....	6	4	5
Saturated.....	39	35	32
Unsaturated.....	61	65	68



## DISCUSSION

Rapid gains in weight during the supplementary feeding period were observed for each of the three groups. The weight gains were greater when the animals received the liver fraction in addition to thiamine, pyridoxine, riboflavin, and choline. The greatest

TABLE VI  
*Distribution of Liver Lipids*

Lipids	No liver fraction		Liver fraction		Liver fraction + lipocaic	
	mg. per single liver	mg. per gm. liver	mg. per single liver	mg. per gm. liver	mg. per single liver	mg. per gm. liver
Acetone-soluble.....	86	27	596	110	106	22
Phospholipid.....	32	10	54	10	48	9.8
Cholesterol, free.....	4.3	1.3	38.5	7.1	4.9	1.0
“ total.....	6.6	2.1	56.3	10.4	6.7	1.4

TABLE VII  
*Effect of Various Dietary Supplements on Increase in Body Weight and Carcass Glycerides*

Supplements	Total gain in weight per animal during supplementary feeding period	Acetone-soluble lipids per animal	Gain in acetone-soluble lipids per animal	
	gm.	gm.	gm.	per cent total gain
None*.....		0.84		
Thiamine only*.....	6	2.31	1.47	24.5
Vitamins B <sub>1</sub> + B <sub>6</sub> + flavin + choline				
No liver fraction....	12	3.56	2.72	24.0
Liver fraction.....	33	7.36	6.52	19.8
“ “ + lipocaic.....	41	9.33	8.49	20.5

\* Data taken from previous publication (1).

weight gain was made by the group which received the pure vitamins, liver fraction, and the pancreatic extract.

The portions of the weight gains due to increases in body fat have been calculated from the percentages of fat found prior (1) to and following the supplementary feeding period (Table VII). When only pure vitamin supplements were given either with or

without choline, 24 to 25 per cent of the total weight gain was due to acetone-soluble lipids deposited in the rat bodies. The liver extract, with and without the pancreatic extract, lowered this proportion to 20 per cent. These values stand in marked contrast to earlier results of the proportion of body weight gains due to fat obtained following periods of inanition in which adult rats, fasted to approximately 25 per cent loss in weight, readily deposited huge quantities of fat upon refeeding. When carbohydrate or protein furnished the sole caloric intake, 35 per cent of the gain in weight was due to fat (8), and when corn oil supplied 84 per cent of the total calories, the weight increase due to deposited fat was 53 per cent (11). In the latter experiments, however, the B vitamins were supplied by yeast.

Since the diet was entirely lacking in fat, the increases in body fat shown in Table VII furnish evidence of the synthesis of fat from non-lipid sources. The composition of the fatty acids isolated from the acetone-soluble depot lipids again shows clearly the high proportions of total  $C_{18}$  acids in such synthetic fat (1, 7). It should be noted that synthesis of  $C_{18}$  acids was even greater when pyridoxine, riboflavin, and choline were supplied in addition to thiamine (49.6 per cent) than when the latter was the only supplement (40.7 per cent) (1). While emphasis is given to the synthesis of  $C_{18}$  acids, especially of palmitoleic acid ( $\Delta^9$ ,  $^{10}$ -hexadecenoic acid) since it is found in large proportions only after periods of active fat synthesis from carbohydrate or protein, the formation of oleic acid as the main product is also clearly recognized. The amounts of each of the three major acids, palmitic, palmitoleic, and oleic, in the synthetic fats are listed in Table VIII, the data of which were calculated from the data given in Tables I, II, and IV for the acetone-soluble lipids per 100 gm. of rat body weight. From these figures, the gain in fatty acids per 100 gm. of body weight and the proportion of the total gain accounted for by each major acid may be determined for each supplement. Palmitic and palmitoleic acids account for the following proportions of the total increase in fatty acids.

Supplement	Per cent of total acid increase
Thiamine only.....	45
Vitamins B <sub>1</sub> , B <sub>6</sub> , riboflavin, choline, no liver fraction.....	54
Liver fraction.....	43
“ “ + lipocaic.....	46

While the liver fraction appears to diminish the quantity of  $C_{16}$  acids, it does not do so in terms of absolute amount. It does, however, increase the quantity of  $C_{18}$  acids and alters the ratio of one type of acid to the other.

The marked deposition of acetone-soluble lipids in the rat livers when the beef liver extract was fed without the pancreas extract is shown in Table I. With the liver fraction alone, fatty livers were obtained with 12 per cent total lipid of which 92 per cent was acetone-soluble. The pancreatic extract effectively held the level of liver fat to a normal value, 3 per cent total lipid, only 69 per cent of which was acetone-soluble.

TABLE VIII

*Major Fatty Acids in Carcass Glycerides per 100 Gm. of Rat Body Weight*

Acids	Vitamin B complex-deficient group*	Supplements to basal diet			
		Thiamine only*	Vitamins B <sub>1</sub> + B <sub>6</sub> + flavin + choline		
			No liver fraction	Liver fraction	Liver fraction + lipocaine
	gm.	gm.	gm.	gm.	gm.
Total acids.....	1.06	2.87	4.86	7.72	9.01
Palmitic.....	0.22	0.79	1.58	2.12	2.12
Palmitoleic.....	0.05	0.30	0.73	1.00	1.83
Oleic....	0.58	1.46	1.90	3.87	4.11

\* Data taken from previous publication (1).

The data presented in Table VI reveal the differences among the lipids of the three groups of livers. The effect of the beef liver fraction added to the mixture of vitamins B<sub>1</sub>, B<sub>6</sub>, riboflavin, and choline was to increase both the total acetone-soluble lipids and the total cholesterol per gm. of liver approximately 5 times. On the same basis, the phospholipid remained remarkably constant in amount despite the enormous increase in total lipid, corroborating the experiences of others (12-14). The pancreas extract kept the acetone-soluble lipids and cholesterol at normal values. McHenry and Gavin (4) pointed out the increase of cholesterol in fatty livers arising from supplementary feeding of the beef liver extract. The value obtained here for total cholesterol in a single liver (56.3 mg.) was somewhat higher than McHenry and Gavin

determined (40 mg.) when the beef liver fraction was fed (4). The total effect was, however, of the same magnitude. Change in the total amount of cholesterol due to the beef liver fraction did not appreciably affect the percentage of free cholesterol found, 68 to 70 per cent. While no balance experiments were run on the cholesterol, it appears likely that the large amount of total cholesterol found in the rat livers of the "liver fraction" group actually represents an increased rate of synthesis of cholesterol, especially since the amount of cholesterol in the body has been shown to be increased by the liver fraction (4).

McHenry and Gavin (4) have already pointed out that the fatty livers caused in rats by the beef liver extract are neither cured nor prevented by choline, as is true in the case of fatty livers produced by feeding a thiamine-rich diet to animals which have been on a diet deficient in the vitamin B complex. These results further substantiate the viewpoint (*a*) that different types of fatty livers are formed under the two sets of conditions and (*b*) that the different fatty livers respond to different lipotropic agents. The results also strengthen the claims for the presence of a lipotropic agent, distinct from choline or protein, in the pancreas (4, 5, 15-17).

#### SUMMARY

It has previously been reported (4) that the administration of a beef liver fraction to rats fed a fat-free diet causes intensely fatty livers, preventable by feeding lipocaic but not by choline; these livers contain large amounts of cholesterol, which is less when lipocaic is also given. There is also an increased quantity of lipids in the carcasses. The fatty acids synthesized when thiamine, riboflavin, pyridoxine, and choline are given are largely  $C_{16}$  and  $C_{18}$  acids, the  $C_{16}$  acids being 54 per cent of the total. Further supplementing the diet with the liver fraction causes a greater increase of  $C_{18}$  acids than of  $C_{16}$  acids and also augments the quantity of unsaturated acids. The fatty livers caused by the liver fraction contain large amounts of acetone-soluble fat which is kept at a normal level by simultaneously feeding lipocaic. While the liver fraction causes a marked increase in cholesterol in the liver, there is no significant change in the ratio of free to ester cholesterol (2:1).

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## PHYTASE IN PLASMA AND ERYTHROCYTES OF VARIOUS SPECIES OF VERTEBRATES

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A phytic acid-splitting enzyme, called phytase, was first found in rice bran by Suzuki *et al.* (1), and phytases were found later in various plant materials (2). The occurrence of phytase in the animal kingdom was reported by McCollum and Hart (3), who found phytase activity in the liver and blood of calves. However, Plimmer (4) and other investigators (5) failed to find the enzyme in extracts of intestines, pancreas, kidney, bones, and also the liver and blood of several species of animals. Recently Patwardhan (6) reported some phytase activity in extracts of rat intestines, but little if any activity was found in the intestines of guinea pigs and rabbits and in bone extracts of all animals studied.

After the finding of phytic acid in the bloods of birds and turtles (7), we examined the blood plasmas and cells of fourteen species of animals for phytase activity and undertook a general survey of the properties and kinetics of this enzyme.

### EXPERIMENTAL

*Plasma Phytase*—Blood samples, drawn with a needle and glass syringe from the wing vein of birds, and by heart puncture from rabbits, frogs, snakes, turtles, and fish, were collected in vials containing heparin. Calf blood was obtained at the slaughter-house, defibrinated, and transported quickly to the laboratory. After centrifugation the plasma was kept for 30 minutes in a desiccator over concentrated NaOH to remove most of the CO<sub>2</sub>, and then an exactly measured volume was mixed with an equal volume of a 0.5 per cent solution of pure recrystallized sodium phytate which had been adjusted to pH 7.2. 1 cc. of this plasma-substrate mixture was precipitated immediately with 2 cc. of 20 per cent

trichloroacetic acid, then diluted with 2 cc. of distilled water, and filtered. 0.5 cc. aliquots of the plasma-substrate mixture were placed in a number of 5 cc. volumetric flasks, and these were divided into pairs. To each flask 0.12 cc. of HCl or NaOH solution was added in concentrations varying so as to give a duplicate series of solutions ranging from pH 4 to 9. Finally 1 drop of toluene was added. One sample of each pair was used for measurement of the pH. The other was incubated at 37.5°, usually for 20 hours, and then precipitated with 2 cc. of 20 per cent trichloroacetic acid, made to volume with distilled water, and filtered. Inorganic P was determined in the control sample and in the samples precipitated after the period of incubation.

A somewhat different procedure was followed in another series of experiments in which buffers were used to obtain the desired pH range. 1 cc. aliquots of the plasma-substrate mixture, prepared as described above, and 0.5 cc. of a buffer solution of sodium diethyl barbiturate and sodium acetate (8), were pipetted into 5 cc. volumetric flasks. Varying amounts of 0.1 N HCl were added, calculated to produce a pH series within limits pH 4 to 9. A drop of toluene was added, and distilled water to make the total volume 5 cc. After these were mixed, a small amount of the sample was withdrawn for pH determination, and the remainder was incubated at 37.5°. After suitable time intervals aliquots were precipitated for the determination of the inorganic P.

Phosphatase activity as well as phytase activity was measured in several of the plasmas. For such experiments 0.5 cc. aliquots of plasma were measured into 5 cc. volumetric flasks, followed by 0.5 cc. of a 5 per cent solution of sodium glycerophosphate (Eastman) and 0.5 cc. of the buffer solution described above. Amounts of 0.1 N HCl, calculated to produce the desired pH, and water to give a final volume of 5 cc. were then added. One sample was precipitated immediately, and the others incubated. Because of the effect of glycerol and inorganic phosphate and of the varying substrate concentration on the rate of glycerophosphate splitting, the incubation time was kept as short as possible, so that the average hydrolysis rates reported here approach the initial rates.

In a number of experiments the pH was measured both at the beginning and at the end of the incubation period. It was found that both in the presence and in the absence of added buffer the pH changed less than 0.1 unit in most of the samples studied.

The pH determinations were performed by means of a glass electrode at 38°. Phosphorus was determined according to the method of Fiske and Subbarow (9).

*Phytase in Cells*—Bloods were drawn and the plasma separated as described in the preceding section. After the samples had been washed twice by suspension in 20 volumes of cold isotonic NaCl solution, and centrifuged, the cells were suspended in enough salt solution to make approximately the original volume of blood. The actual percentage of cells in the suspension was determined by the hematocrit method (10). With certain blood cells, particularly those of frogs, difficulties were encountered during the washing process. These cells when washed showed a great tendency to clumping, gelation, and subsequent hemolysis. Pipetting was impossible, and the cell volume could not be determined. Frog cells were therefore washed only once, with a large volume of saline, as quickly as possible.

Suspensions of mammalian erythrocytes were incubated immediately after the washing with saline as follows: two aliquots of 1 cc. each were pipetted into 5 cc. volumetric flasks, followed by 0.1 cc. of a saturated solution of saponin, to hemolyze the cells, and 0.5 cc. of the stock buffer solution and 1.45 cc. of 0.1 N HCl to produce a pH of approximately 6.6, at which the phytase activity is maximal. To one flask were added 1 cc. of a 0.5 per cent solution of sodium phytate and distilled water to give a final volume of 5 cc. To the other flask serving as a control only distilled water was added. Both flasks were incubated for about 20 hours at 37.5°. An aliquot was then deproteinized with trichloroacetic acid and the inorganic P determined. Any increase in the concentration of inorganic P in the sample which had been incubated in presence of phytate, compared with the control sample, was assumed to be due to the action of phytase.

Suspensions of frog and snake blood cells were subjected to preliminary incubation with 0.0025 N KCN for 4 hours at 37.5°, to liberate most of the inorganic P of the adenosine triphosphate, and then they were treated as described in the preceding paragraph.

The presence of phytic acid in the blood cells of birds and turtles did not permit the procedure described above. Therefore another method was adopted, based on the finding that intact cells in the presence of KCN showed a rapid increase of inorganic P owing to the breakdown of organic acid-soluble phosphorus compounds



other than phytic acid. A suspension of cells was prepared as described in the first paragraph of this section, its volume measured, and an amount of 0.05 N KCN solution calculated to give a concentration of approximately 0.0025 N KCN was added. The per cent volume of cells was then determined, and the suspension was incubated for 5 hours at 37.5°. During this period organic acid-soluble P other than phytate was decomposed. After the incubation 0.5 cc. aliquots of the suspensions were distributed into a series of 5 cc. volumetric flasks, and 0.1 cc. of a saturated solution of saponin and 0.5 cc. of the stock buffer solution added. Appropriate amounts of 0.1 N HCl to produce a series ranging from pH 4 to 9 and distilled water to give a final volume of 5 cc. were

TABLE I

*Comparison between Increase of Inorganic P and Decrease of Phytate P in Hemolysates of Goose Erythrocytes Previously Incubated for 6 Hours in Presence of 0.0028 N Cyanide*

Incubation time 39 hours at 37.5°; cell volume 27.7 per cent.

pH	Inorganic P	Increase	Phytate P*	Decrease
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
	10.7		18.8	
4.85	12.4	1.7	17.6	1.2
6.86	16.9	6.2	13.0	5.8
7.45	16.6	5.9	12.0	6.8
8.13	13.7	3.0	15.7	3.1
9.36	11.5	0.8	18.2	0.6

then added. One sample, precipitated immediately, was taken as the blank. The others were incubated at 37.5°, then precipitated with trichloroacetic acid, and the inorganic P determined. Any increase in the inorganic P compared with the blank value was assumed to be due to phytase activity. The validity of this method was checked by direct determination of the disappearance of phytic acid. For such experiments 2 cc. aliquots of the erythrocyte suspensions were taken for determination of phytate P by the method of Leva and Rapoport (unpublished work) at the beginning and at the end of the incubation period and the results compared with the simultaneously determined inorganic P (Table I). The results showed that the determination of increases of

inorganic P in hemolysates of bird and turtle erythrocytes, after a preliminary period of incubation in the presence of cyanide, was an adequate measure of the decomposition of phytic acid.

### Results

*Plasma*—The pH-activity curves of phytase in the plasmas of goose, pigeon, chicken, turtle, and frog bloods are shown in Fig. 1. The curves on the whole have similar shapes. The pH at which

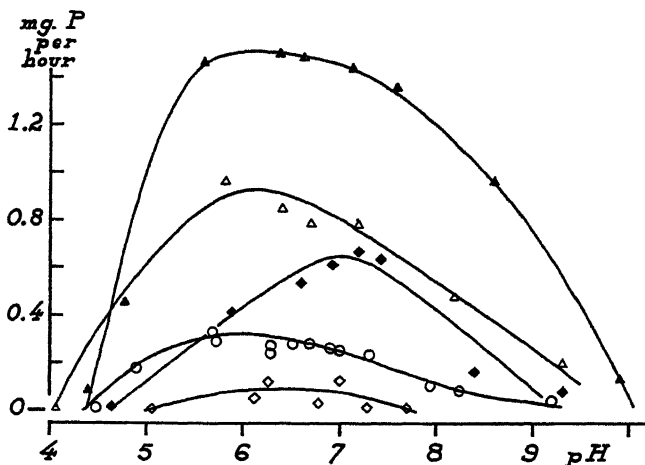


FIG. 1. pH-activity curve of phytase of plasma. The enzyme activity is expressed in mg. of P liberated by 100 cc. of plasma in 1 hour. ▲ goose, △ pigeon, ◆ turtle, ○ frog, ◇ chicken.

maximal activity occurred was found to vary considerably both in different specimens of one species and in plasmas of different species. The range of this variation was from pH 5.7 to 7.2, the average pH for the maximal activity being 6.6 for all specimens studied. On the acid side of the maximum the activity was found to decrease very rapidly below pH 5. On the alkaline side of the maximum a more gradual diminution took place, with some activity even above pH 9. The curve of chicken plasma was difficult to interpret on account of its low phytase activity. The maximal activity appeared to occur near pH 7.

The time-activity curve of phytase in goose plasma, shown in Fig. 2, indicated that the decomposition of phytate proceeded at a

constant rate. This indicated that the enzyme was insignificantly inhibited by the split-products of the substrate. The linearity of the relation between time and the amount of phytate split permitted the expression of the activity of the enzyme in terms of mg. of phytate P split by 100 cc. of plasma in 1 hour.

*Factors Influencing Phytase Activity*—In these experiments 0.5 cc. aliquots of plasma-substrate mixture were measured into 5 cc. volumetric flasks, followed by 0.12 cc. of HCl calculated to give a pH of approximately 6.6, and 0.38 cc. of a solution of the substance to be tested was added. The mixtures were incubated at 37.5° and the amount of phytate P split was compared with that of

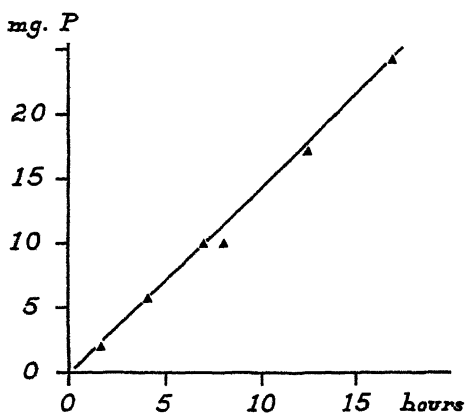


FIG. 2. Time-activity curve of phytase of goose plasma

a control sample to which 0.38 cc. of distilled water had been added. The pH of the mixture was determined in a duplicate sample similarly treated.

Table II summarizes the effects of various agents on the activity of phytase in goose plasma. The data show that neither phosphate nor inositol, the split-products of phytic acid, had an appreciable effect on enzyme activity. Addition of Mg ions did not increase the activity of the enzyme. The activity was found to be inhibited by fluoride and oxalate ions, oxalate exerting the more pronounced effect.

Table III shows that the phytase in goose plasma was singularly insensitive to changes in substrate concentration, indicating a very

low value for the dissociation constant of the substrate-enzyme complex.

TABLE II

*Effect of Various Agents on Phytase Activity of Goose Plasma*

0.25 cc. of goose plasma, 0.25 cc. of 0.5 per cent sodium phytate, 0.12 cc. of 0.025 N HCl, and 0.38 cc. of a solution of the agent to be tested were incubated at 37.5° for 18 hours; pH 6.6.

Added		P liberated by 100 cc. plasma per hr.	Inhibi- tion	Added		P liberated by 100 cc. plasma per hr.	Inhibi- tion
	mg. per 100 cc.	mg.	per cent		mg. per l.	mg.	per cent
Phosphate*	0	1.40	0	MgCl <sub>2</sub>	0	1.38	0
	10	1.28	9		1	1.31	5
	30	1.24	11		3	1.31	5
	100	1.29	8		10	1.30	6
Inositol	0	1.40	0	NaF	30	1.27	8
	90	1.31	6		0	1.38	0
	360	1.38	2		20	1.18	16
					80	0.79	44
				Na oxalate	0	1.12	0
					10	0.47	58
					25	0.34	70

\* Mixture of Na<sub>2</sub>HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub>, pH 7.0.

TABLE III

*Effect of Substrate Concentration on Activity of Phytase of Goose Plasma*  
pH 6.4; incubation time 5 hours.

Concentration of phytate	P split by 100 cc. plasma per hr.
mg. per 100 cc.	mg.
6	1.40
11	1.40
20	1.41
35	1.42

The effect of high temperatures on the phytase of goose plasma is shown in Table IV. In these experiments plasma samples, previously adjusted to pH 6.6, were heated for 10 minutes in a water bath at the various temperatures given in Table IV. After

the samples were cooled, the substrate was added, and the mixtures were incubated 10 hours at 37.5°. The rates of phytate splitting in the various samples were compared with that of a control sample of plasma which had not been heated. The data show that heating to 50° for 10 minutes perceptibly depressed the phytase activity and that at 60° 87 per cent of the enzyme was destroyed.

The phytase activities of the plasma of various species are compared in Table V. The values reported serve only as a rough indication of species differences, since in many instances only a single specimen of plasma of a species was studied. The data on plasmas of geese, the most extensively studied, may perhaps furnish an estimate of the variation to be expected among individuals of a species. However, much wider differences were found among

TABLE IV

*Effect of Heating on Activity of Phytase of Goose Plasma*

Plasma adjusted to pH 6.4 was heated for 10 minutes in a water bath at the temperatures given, cooled, then the substrate was added, and the mixture incubated at 37.5° for 10 hours.

	Un-heated	50°	60°	70°	80°	90°
P split by 100 cc. plasma per hr., mg.....	1.10	1.02	0.14	0.04	0.00	0.00
Inactivation, per cent .....	0	8	87	96	100	100

different species. Among the birds, in many respects a very uniform group, the plasma phytase was found to vary from 0.07 to 0.11 mg. per hour in chicken plasma, and from 0.99 to 1.57 mg. in goose plasma. Similarly the two species of fish studied showed widely differing enzyme activities. All mammalian plasmas studied were found free of phytase activity. In some of the plasmas the activity of the alkaline phosphatase was compared with the phytase activity (with glycerophosphate as substrate).<sup>1</sup> All mammalian plasmas, devoid of phytase, showed an active alkaline phos-

<sup>1</sup> In some plasmas more complete pH-activity curves of phosphatase activity, with glycerophosphate as substrate, were made. These are omitted here for the sake of brevity. Only frog plasma gave evidence of the presence of a weak acid phosphatase, in addition to alkaline phosphatase.

phatase. Among the lower vertebrates great differences in the distribution of the two enzymes were found. Chicken plasma, the lowest in phytase activity, exhibited a highly active alkaline phosphatase, whereas the plasma of bass showed the highest phytase and the lowest phosphatase activity of all plasmas.

TABLE V

*Phytase and Phosphatase Activities of Plasmas of Various Species*

The activities were determined at pH 6.6, assumed to be the pH of maximum activity, in all cases except the plasmas of goose, pigeon, chicken, snapping turtle, and bullfrog. For the latter, the values were determined at the pH actually found to give maximum activity.

Species	P split by 100 cc. plasma per hr.	
	Phytase	Phosphatase
	mg.	mg.
Goose	0.99-1.57 (1.22 average)	1.68
Pigeon	1.05	0.36
Duck	0.47	
	0.58	
Chicken	0.07	0.88
	0.11	1.10
Turtle	0.71	0.90
Moccasin snake	0.25	9.5
Brown water-snake	0.12	7.5
Bullfrog	0.27	0.63
	0.33	0.40
Bullhead	0.70	0.30
Black bass	1.73	0.30
Man	<0.03	1.60
Rabbit	<0.03	1.26
Guinea pig	0	1.40
Beef	0	
Calf	0	

*Cells*—The relationship between pH and phytase activity of the erythrocytes of pigeon, goose, chicken, and turtle is shown in Fig. 3. Inspection of the figure shows that the values from different species fall almost entirely on a single curve. The maximum activity of pigeon, chicken, and goose erythrocytes was found at pH 6.8 to 6.9, but was at a greater acidity in turtle erythrocytes. The comparison of the curve of cell phytase with

that of plasma phytase shows the left-hand side of the cell curve to be somewhat flatter. The significance of this difference is doubtful.

In Table VI is represented the time-activity relationship of the phytase of goose erythrocytes, apparently linear within the limits of error.

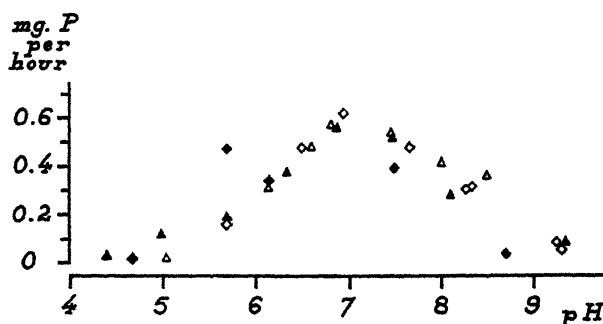


FIG. 3. pH-activity curves of phytase of the blood cells of ▲ goose, △ pigeon, ◇ chicken, ◆ turtle. The enzyme activity is expressed in mg. of P liberated by 100 cc. of cells in 1 hour.

TABLE VI

*Time-Activity Relationship of Phytase of Goose Erythrocytes*

Cell volume 50.2 per cent; temperature 37.5°; pH 6.6. The hydrolysis rate was measured by determination of the decrease in phytate.

Time	Phytate P	Difference	P split by 100 cc. cells per hr.
hrs.	mg. per 100 cc.	mg. per 100 cc.	mg.
0	33.9		
10	30.9	3.0	0.59
21	27.7	6.2	0.59
48	19.3	14.6	0.61
76	10.8	23.1	0.60

In Table VII is shown the inhibitory effect of oxalate on phytase in the erythrocytes of goose, chicken, and turtle. The inhibition was apparently of the same degree as was found in plasma.

Thus the available facts suggest strongly the identity of the enzyme occurring in plasma and erythrocytes.

In Table VIII the data on the activity of cell phytase of various species are summarized. No enzyme activity was found in the

cells of the mammals studied, or in the cells of frogs and snakes. Thus the occurrence of phytase in erythrocytes, as far as investigated, seemed limited to those which contain phytic acid. Particularly remarkable is the fact that the phytase activity was of the same order of magnitude in all erythrocytes containing the

TABLE VII

*Inhibitory Effect of Oxalate on Activity of Phytase of Erythrocytes of Various Species*

Sodium oxalate was added to give a final concentration of 0.024 N, and the samples were incubated for 24 hours at 37.5°; pH 6.6.

Species	P split by 100 cc. cells per hr.		
	Control	Oxalate added	Inhibition
	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Goose	0.59	0.18	70
Chicken	0.57	0.13	77
Turtle	0.52	0.13	75

TABLE VIII

*Activity of Phytase of Erythrocytes of Various Species*

The activities were determined at the pH of maximum activity for each species.

Species	P split by 100 cc. cells per hr.
	<i>mg.</i>
Goose	0.56-0.59 (0.58 average)
Pigeon	0.62
Chicken	0.59
	0.64
Turtle	0.52

The blood cells of frog, moccasin snake, brown water-snake, rabbit, guinea pig, beef, calf, and man were tested for phytase activity at pH 6.4 with negative results.

enzyme. This is in contrast with the great variation found in the activity of phytase among various plasmas.

#### DISCUSSION

Owing to the limited material of this study only preliminary conclusions may be formulated. Among the species examined,



phytase is absent in the cells and plasmas of the mammals and is present in the plasmas of the lower vertebrates. The occurrence of phytase in erythrocytes seems limited to those which contain phytic acid. In these the activity of the enzyme is characterized by remarkable constancy compared with the great variability found in plasmas. Further studies are necessary to establish the significance of phytase and of its substrate in animal metabolism.

Phytase appears to be a separate entity, distinct in nature from other phosphatases. The enzymes in plasma and cells appear to be identical, as judged by pH-activity curves, the time-activity curves, and other characteristics.

The only previous study of phytase in which the properties of the enzyme in animal tissues were studied was made by Patwardhan (6). In several respects his results, obtained with an enzyme preparation from rat intestine, differ from those described here. Patwardhan found a more alkaline pH optimum for the enzyme preparation from rat intestine, and reported activation of the enzyme by Mg ions. Further studies on phytase in various tissues may show whether there exist several enzymes capable of splitting phytic acid, but differing in their properties.

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# PHOSPHORUS EXCHANGE IN PHYTATE, LIPIDS, AND NUCLEOPROTEINS IN THE ERYTHROCYTES OF GEESE\*

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After the finding of phytic acid in avian erythrocytes (1) we undertook studies of the intermediary metabolism of this compound in the erythrocytes of geese. In preliminary experiments goose blood was incubated aerobically and anaerobically, alone and in the presence of fluoride, iodoacetate, arsenate, and other substances known to affect reactions of the glycolytic cycle in blood, but no significant changes in the concentration of phytate were found in the bloods thus treated. Believing that the failure to demonstrate changes in the phytate under such conditions might be due to a very slow turnover of this compound or to a close balance between its synthesis and decomposition, we next used radioactive phosphorus as a tracer. The studies were then extended to include the phospholipids and nucleoproteins.

## EXPERIMENTAL

Blood was drawn from the wing veins of geese and collected in vials containing 1 mg. of heparin per 5 cc. of blood. Crystalline heparin was obtained from the Connaught Laboratories, Toronto, Canada. Radioactive phosphorus was added to the blood samples in the form of  $\text{Na}_2\text{HPO}_4$ , dissolved in 0.9 per cent NaCl solution.

For experiments performed under aerobic conditions approximately 4.5 cc. of blood were delivered into a 50 cc. flask. After

\* The radioactive phosphorus used in these studies was supplied by the Department of Physics, The University of Rochester, Rochester, New York, through the courtesy of Dr. L. A. DuBridge and Dr. S. N. Van Voorhis.

addition of the radioactive P a current of oxygen was passed through the flask 2 minutes, with constant shaking, and the flask was then stoppered. The amount of oxygen present proved sufficient to keep the blood oxygenated during the entire period of incubation in all experiments.

Anaerobic conditions were obtained in three ways. (1) A solution of KCN (0.05 N in 0.9 per cent NaCl solution) was added to the blood in sufficient amount to give a concentration of approximately 0.003 N KCN. (2) The blood was saturated with carbon monoxide. For this 4.5 cc. of blood were placed in a 50 cc. centrifuge tube fitted with a 2-hole stopper. The tube was evacuated and then CO gas was passed through the tube for 2 minutes with constant shaking of the sample. The holes of the stopper were then closed and the sample was incubated in the presence of the CO gas in the tube. (3) Blood was incubated in an atmosphere of nitrogen. The sample was placed in a thick walled 250 cc. Erlenmeyer flask fitted with a 2-hole rubber stopper. Through delivery tubes the flask was alternately evacuated and flushed with nitrogen gas four times. The sample then had assumed a dark purple color which did not deepen visibly during subsequent incubation.

The blood samples thus prepared were incubated at 37.5° for the periods of time indicated in Table I. The aerobic samples were rotated continuously during the incubation period. After the incubation 3 cc. of blood were pipetted into a 50 cc. centrifuge tube containing 6 cc. of 20 per cent trichloroacetic acid and 6 cc. of water were added. After thorough mixing the tube was centrifuged and the supernatant fluid containing acid-soluble P compounds was drawn off for the analyses described in the next paragraph. To remove the remainder of the acid-soluble P from the precipitate, the precipitate was washed twice by suspension and centrifugation with 20 cc. of 5 per cent trichloroacetic acid containing 10 mg. of inorganic P per 100 cc. and twice with phosphorus-free 5 per cent trichloroacetic acid. For extraction of the phospholipids the washed precipitate was suspended in 25 cc. of a 3:1 alcohol-ether mixture, centrifuged, and the supernatant liquid drawn off. The precipitate was resuspended in 1 cc. of water, again extracted with 25 cc. of the alcohol-ether mixture, centrifuged, and the supernatant fluid combined with the first alcohol-ether extract. These combined extracts were then evaporated,

ashed with sulfuric and nitric acids, and made to a definite volume. After extraction of the phospholipids the residue containing the nucleoproteins was thoroughly dried, dissolved in a few cc. of approximately 8 N nitric acid in a boiling water bath, transferred quantitatively to a 10 cc. volumetric flask, and made to volume.

Determinations of the total acid-soluble P and phytate P were made on the first trichloroacetic acid extract. To obtain the phytate fraction uncontaminated by other radioactive fractions of the acid-soluble P the following procedure was followed. Magnesia mixture and ammonium hydroxide were added to 10 cc. of the extract, representing 2 cc. of original sample, to precipitate inorganic P and phytate, according to the method of Leva and Rapoport (unpublished). After centrifugation the precipitate was dissolved in 2.5 cc. of N HCl, boiled 10 minutes to decompose any coprecipitated adenosine triphosphate, diluted with an equal amount of distilled water, and then 5 cc. of a 25 per cent solution of calcium acetate were added to precipitate the phytic acid. The precipitate was dissolved in 5 cc. of 0.5 N HCl containing 10 mg. of inorganic P per 100 cc., and reprecipitated with an equal volume of 25 per cent calcium acetate. The dissolving of the precipitate in HCl and reprecipitation with calcium acetate were repeated once more with phosphorus-containing HCl solution and twice with a phosphorus-free solution. Finally the precipitate of calcium phytate was dissolved in dilute HCl solution and made to suitable volume. In control studies of this procedure with solutions of pure phytic acid 85 per cent of the phytate P was recovered. The values for phytate P listed in Table I were corrected accordingly. The phosphorus content of the various fractions was determined by the method of Fiske and Subbarow (2). The radioactivity was measured by means of a Geiger-Müller counting apparatus assembled for us by Dr. W. F. Bale of the Department of Radiology, The University of Rochester (3).

In a comparison of the radioactivities of the various classes of phosphorus compounds the assumption was made that all acid-soluble P other than phytate P had exchanged completely and within a comparatively short time with the inorganic P in the serum. The validity of this assumption is discussed later. The radioactivities per mg. of phytate P, lipid P, and nucleoprotein

P were therefore expressed in percentages of the radioactivity per mg. of acid-soluble P other than phytate P.

### Results

The results of the experiments listed in Table I indicate that in blood incubated aerobically the exchange of phytate P was slow

TABLE I

*Exchange of Radioactive Phosphorus in Phosphorus Compounds of Goose Blood Incubated at 37.5°*

Ex- peri- ment No.	P <sup>32</sup> added to blood	Condi- tions	Time of in- cuba- tion	Phy- tate P	Lipoid P	Protein P	Acid- soluble P other than phytate P	Radioactivity per mg. P compared with that of acid-soluble P other than phytate P		
								Phy- tate	Lipid	Pro- tein
	kicks per min. per mg. P		hrs.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	per cent	per cent
1	4,000	O <sub>2</sub>	24	33.5			27.5	2.50		
	4,000	KCN	24	34.0			27.5	0.03		
	4,000	"	48	33.3			27.5	0.01		
2	825	O <sub>2</sub>	22	30.1			17.0	2.17		
	825	KCN	22	30.2			17.0	0.18		
3	8,850	O <sub>2</sub>	24	22.2	13.0	66.7	22.0	2.72	1.79	0.89
	8,850	KCN	24	22.7	13.0	67.3	21.6	0.03	0.08	0.08
4	9,810	CO	24	21.1	11.8	65.1	21.0	0.001	0.000	0.001
	9,810	N <sub>2</sub>	24	20.7	11.8	65.2	21.4	0.019	0.008	0.010
5	18,450	A*		14.1	10.2	46.6	19.8	1.38	1.46	0.72
	18,450	B		12.8	10.2	45.2	21.2	1.29	1.50	0.72
	18,450	C		12.1	10.2	42.3	22.3	0.25	0.49	0.22
6	7,590	†		16.2	10.4	47.6	27.8	1.41	1.25	0.74

\* After incubation for 1 hour (A), 3 hours (B), and 14 hours (C) in N<sub>2</sub> atmosphere the blood was incubated 12 hours aerobically.

† After incubation for 1 hour in the presence of 0.003 N KCN the blood cells were washed four times with ice-cold saline, then resuspended in saline plus P<sup>32</sup> and glucose, and incubated for 12 hours in the presence of oxygen.

compared with the exchange in other acid-soluble P compounds. The turnover of phosphorus in the phospholipids and proteins was approximately the same as it was in the phytate. Under conditions of anaerobiosis obtained by the addition of KCN to the blood or by incubation in an atmosphere of carbon monoxide or

nitrogen the slow turnover of phosphorus in the phytate, phospholipids, and nucleoproteins was almost completely inhibited.

Experiments 5 and 6 represented in Table I were designed to determine whether the inhibitory effect of anaerobiosis on the turnover of phosphorus in the stable compounds was reversible. In these experiments blood samples were incubated anaerobically for varying periods of time and then oxygenated and incubated aerobically as in the other experiments. The results indicate that the effects of incubation in an atmosphere of nitrogen for 1 to 3 hours were completely reversible. The effects of 14 hours incubation were, however, only partly reversible, since the rate of exchange following this period of anaerobiosis was only one-fourth of that found after the shorter periods of anaerobic incubation. Experiment 6 yielded results indicating that the effect of cyanide also was reversible. In this experiment blood was incubated in the presence of 0.003 N KCN for 1 hour and the cells were then washed four times with large amounts of cold 0.9 per cent NaCl solution. The cells were then suspended in 0.9 per cent NaCl solution containing 10 mg. of inorganic P and 300 mg. of glucose per 100 cc., radioactive phosphorus was added, and the suspension was incubated aerobically.

#### DISCUSSION

The findings here reported indicate that a slow turnover of phytic acid as well as of the phospholipids and nucleoproteins in the erythrocytes of goose blood takes place during incubation under aerobic conditions *in vitro*. Thus phytic acid, an acid-soluble compound of comparatively small molecular weight, falls in the class of the so called structural or stable phosphorus compounds. The great stability of these compounds under aerobic conditions suggests that both synthesis and degradation of these substances are dependent upon aerobic metabolism of the cells. It would appear that in the respiring cell a dynamic equilibrium, perhaps better called a steady state, is maintained by continuous formation and decomposition of the structural compounds; whereas with fermentation under anaerobic conditions a similar balance is maintained statically in the cells, owing to the absence of anabolic and catabolic processes which affect the stable compounds.

For purposes of comparison the assumption was made that all acid-soluble P other than phytate P had exchanged completely and quickly with the inorganic P of the serum. It may be useful to enumerate here some of the factors which might affect this estimate of the rate of turnover of the stable phosphorus compounds and to indicate the direction in which the results here reported might be altered. (1) Any correction that might be applied for the rate of penetration of inorganic P into the cells would tend to lower the average radioactivity of the acid-soluble P other than phytate P during the experiment and thus increase proportionately the estimate of the turnover of P in the stable phosphorus compounds. (2) The presence in the acid-soluble P other than phytate P of compounds with a lower exchange rate would tend to increase the average value for the radioactivity of the inorganic P in the cells, thus having the opposite effect on our estimate of the turnover of stable phosphorus compounds. (3) The whole basis of comparison is affected by the lack of knowledge of the fraction or fractions of the acid-soluble P with which the stable phosphorus compounds exchange. If, for instance, the phospholipids of the cell exchanged preferentially with the inorganic P in the serum, the turnover of P in this fraction would occur at a much lower rate than is indicated by our mode of comparison. On the other hand, if this fraction exchanged with a compound of moderately low activity, our estimate of the turnover would be increased considerably. The rates given in Table I appear to be valid for purposes of comparison in this preliminary survey of the problem. Further studies will be required to establish appropriate bases of comparison for each class of phosphorus compounds.

Further studies of the slight changes in concentration of the stable phosphorus compounds found to occur under conditions of these experiments will be reported and discussed in a later communication. The slowness of the turnover of phosphorus in these compounds, as well as the delicate balance between their anabolism and catabolism, so closely maintained, necessitates sensitive methods for their study. The use of radioactive indicators appears to be the most sensitive method available for elucidating the intermediary metabolism of such compounds.

## SUMMARY

With radioactive phosphorus as a tracer, a slow aerobic turnover of phosphorus was found in phytate, lipids, and nucleoproteins in the erythrocytes of geese *in vitro*. The phosphorus exchange was inhibited reversibly by anaerobic conditions obtained by means of cyanide, carbon monoxide, and nitrogen.

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## INSULIN AZO DERIVATIVES

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In attempts to study the nature of groups essential for insulin activity a number of various derivatives of insulin have been prepared. The reagents (halogens, alkyl and acyl halides, aldehydes, aryl isocyanates (1-3), carbobenzoxy chloride (4), and O- $\beta$ -glucosidocarbobenzoxytyrosine azide (5)) were as a rule used in excess and the result was a complete or almost complete inactivation of the insulin. In some instances in which the reaction could be reversed the physiologic activity could be at least partially recovered. Gaunt and Wormall (4) have treated insulin with various amounts of carbobenzoxy chloride and found that inactivation was only partial if the amount of reagent was not more than equivalent to the free amino groups of insulin.

Aryldiazonium chlorides which have been used extensively as reagents with proteins in immunochemical studies (6) have according to our knowledge not been used for the preparation of insulin derivatives. Their use is particularly convenient for the purpose of introducing strongly acidic and basic groups into a protein. The preparation of derivatives of insulin containing basic groups is of interest also because it has been demonstrated recently that the hypoglycemic activity of insulin is considerably prolonged by the formation of addition compounds of insulin which are only slightly soluble at neutral pH. The investigation of the hypoglycemic activity of synthetic insulin derivatives having a different precipitation zone from that of insulin was in part the purpose of this study.

### EXPERIMENTAL

During the past 5 years we have prepared a considerable number of insulin azo dyes. The radicals coupled to insulin were selected

in such a manner as to allow for the comparison of effects produced by anionic and cationic groups and of groups which were similar from an electrochemical point of view but different in weight and which sometimes possessed a structure known to convey surface-active properties to a molecule of which they are a part.

The compounds which were chosen for representation of the group in this paper are insulin derivatives prepared with salts of *p*-diazobenzenesulfonic acid,  $\omega$ -*p*-diazophenylcaproic acid, *p*-diazobenzyltrimethylammonium chloride, and  $\omega$ -*p*-diazophenylamyltrimethylammonium chloride.<sup>1</sup>

The diazonium salts were prepared in the usual way, with the theoretical amount of sodium nitrite in aqueous acid medium. Sometimes alcohol-water mixtures were used as solvents and in rare instances diazotization was carried out in alcohol with amyl nitrite. The diazonium salts were sometimes isolated and recrystallized; more frequently they were used without any further purification. Their concentration was, however, determined by comparing the color intensity produced when they were coupled to  $\beta$ -naphthol with a standard solution of the corresponding azonaphthol derivative.

Coupling was carried out at ice box temperature either by adding to the original acid mixture of insulin and diazonium salt enough 0.1 N NaOH to bring the pH to 8 to 9 or in phosphate buffer of pH 7. The mixture was allowed to stand until all diazonium salt disappeared. The insulin used was Wellcome Brand in crystalline form. The number of groups introduced into the insulin will be expressed as groups per mole, the round value of 40,000 gm. being taken as the molecular weight of insulin.<sup>2</sup> All insulin dyes were brownish yellow in acid and brownish red in alkali solution. The relative color intensities determined in the colorimeter were found to be proportional to the number of groups coupled, but compounds obtained by coupling in phosphate buffer

<sup>1</sup> We are indebted to Dr. J. S. Buck for the preparation of three of the aniline derivatives used in this work. Acknowledgment is also due to Dr. D. S. Searle for his assistance.

<sup>2</sup> Cohn, Ferry, Livingood, and Blanchard (7) discuss various determinations of the molecular weight of insulin. All reliable values which were obtained between 1935 and 1941 fall within the range of 37,000 to 42,000, the most probable value being 40,900.

were not comparable with those which were coupled in dilute NaOH, the former being of slightly different tint and also less intense in color.

The isoelectric precipitation zones of these derivatives were determined in a buffer mixture of 0.2 M  $\text{Na}_2\text{HPO}_4$  and 0.1 M citric acid. The pH values were checked electrometrically and adjusted when necessary. Insulin was added to these buffer mixtures in the form of a hydrochloride solution. The final concentration of

TABLE I  
*Isoelectric Precipitation Zone of Insulin Azo Derivatives*

Insulin derivatives	No. of radicals per molecule	pH*						
		2.0	3.0	4.0	5.0	6.0	7.0	8.0
<i>p</i> -Azobenzenesulfonic acid. . . . .	3	—	—	+	2+	±	—	—
“ “ “ . . . . .	6	—	±	+	2+	±	—	—
“ “ “ . . . . .	15	±	2+	2+	2+	±	—	—
$\omega$ - <i>p</i> -Azophenylcaproic acid. . . . .	3	—	—	+	2+	±	—	—
“ “ “ . . . . .	6	—	±	+	2+	+	—	—
“ “ “ . . . . .	15	—	+	2+	2+	2+	—	—
<i>p</i> -Azobenzyltrimethylammonium chloride. . . . .	3	—	—	+	2+	±	—	—
“ “ “ . . . . .	6	—	—	+	2+	+	±	—
“ “ “ . . . . .	15	—	—	2+	2+	2+	2+	2+
$\omega$ - <i>p</i> -Azophenylamyltrimethylammonium chloride	3	—	—	±	+	±	—	—
“ “ “ . . . . .	6	—	—	±	2+	+	±	—
“ “ “ . . . . .	15	—	—	2+	2+	2+	2+	2+
Crystalline insulin. . . . .		—	—	±	+	—	—	—

\* — = clear, ± = very slightly turbid, + = slightly turbid, 2+ = turbid.

insulin was 1.5 units per cc. Readings were made after the mixtures had stood for 30 minutes at room temperature. (*Cf.* Table I.)

The physiologic activity of the compounds was tested within a few weeks after preparation by the usual cross-over method on fasting rabbits of about 3 kilos in weight ( $2 \times 30$ ). Amounts corresponding to 0.50 unit of insulin were injected per kilo, except when attempts were made to determine the deterioration of insulin activity in dyes containing fifteen cationic groups. In this case,

1.5 units of the insulin dye were compared with 0.5 unit of insulin. In some experiments, as indicated in Table II, zinc was added to both insulin and the insulin derivative in amounts corresponding to 0.3 mg. of zinc per 100 units of insulin or its equivalent. This

TABLE II

*Hypoglycemia Produced after Injection of Insulin Azo Derivatives*

The blood sugar values are given in mg. per cent.

Insulin derivatives	No. of radicals per molecule	Average blood sugar				
		0	1½ hrs.	3 hrs.	5 hrs.	7 hrs.
<i>p</i> -Azobenzenesulfonic acid . . . . .	6	95.7	61.0	64.5	83.6	93.1
Crystalline insulin . . . . .		95.9	56.1	65.3	91.1	97.7
<i>p</i> -Azobenzenesulfonic acid* . . . . .	15	94.4	66.5	73.8	91.9	96.8
Crystalline insulin* . . . . .		94.0	63.7	76.6	93.1	97.1
$\omega$ - <i>p</i> -Azophenylcaproic acid . . . . .	6	100.5	69.7	70.5	89.0	97.4
Crystalline insulin . . . . .		101.4	64.0	68.0	90.5	102.5
<i>p</i> -Azobenzyltrimethylammonium chloride . . . . .	6	92.8	66.6	67.8	79.7	87.4
Crystalline insulin . . . . .		94.9	58.0	61.0	79.3	94.7
$\omega$ - <i>p</i> -Azophenylamyltrimethylammonium chloride . . . . .	3	97.6	65.4	68.8	89.5	98.4
Crystalline insulin . . . . .		97.2	59.1	65.0	88.9	100.5
$\omega$ - <i>p</i> -Azophenylamyltrimethylammonium chloride . . . . .	6	96.8	69.5	72.9	89.2	97.1
Crystalline insulin . . . . .		94.9	64.3	64.3	82.7	94.7
$\omega$ - <i>p</i> -Azophenylamyltrimethylammonium chloride* . . . . .	15	99.0	78.4	83.2	93.9	94.8
Crystalline insulin* . . . . .		99.0	64.3	72.3	90.7	95.6
$\omega$ - <i>p</i> -Azophenylamyltrimethylammonium chloride*† . . . . .	15	96.8	64.4	67.4	77.2	87.3
Crystalline insulin* . . . . .		98.3	61.6	69.2	92.7	98.3

\* Without added zinc. To all other preparations 0.3 mg. of zinc was added for amounts corresponding to 100 units of insulin.

† Amounts corresponding to 1.5 units per kilo. All other preparations were given in amounts corresponding to 0.5 unit per kilo.

was done to minimize the effect of possible loss of zinc from the insulin during the preparation of the derivative.

The blood sugars were determined by the Hagedorn-Jensen method. The relative potencies were calculated as described by Hershey and Lacey (8). This method of calculation is correct

only if the two blood sugar time curves are practically identical. This was not always the case in our experiments. (Cf. Table II.)

*Insulin p-Azobenzenesulfonic Acid*—Dyes containing three, six, and fifteen groups were prepared. They all showed a widened isoelectric precipitation zone when compared with crystalline insulin. The extension of the precipitation zone increased progressively towards the acid side as an increasing number of groups were introduced into the molecule. The insulin derivative which contained fifteen azobenzenesulfonic groups was not entirely soluble at pH 2. The blood sugar time curves obtained with these insulin derivatives were slightly flatter than those obtained with insulin. There was no loss in potency. The dye containing six groups per molecule as well as the dye containing fifteen groups per molecule showed a potency of 1.04 units per unit of original insulin. (Cf. Table II.)

*Insulin  $\omega$ -p-Azophenylcaproic Acid*—The dyes containing three, six, and fifteen groups showed a slightly extended precipitation zone towards the alkaline and a markedly extended zone towards the acid side of the isoelectric point of insulin. The dye containing fifteen groups gave definite precipitation at pH 3 to 6, while insulin under similar conditions precipitated at pH 5 only, and showed a very slight turbidity at pH 4. Only the dye containing six groups was tested for hypoglycemic activity. The results given in Table II again show some flattening of the blood sugar time curve as compared with that for crystalline insulin and apparently also some loss of potency.

*Insulin p-Azobenzyltrimethylammonium Chloride*—The precipitation of these dyes was only slightly extended towards the acid side of the isoelectric point of the insulin but considerably towards the alkaline side, so that the dye containing fifteen groups was almost insoluble at pH 8. The test on rabbits showed that the blood sugar time curves were flatter with these derivatives than those obtained with derivatives having the same number of anionic groups. There was a loss in activity if the potency was estimated on the basis of values observed up to 5 hours after injection. (Cf. Table II.)

*Insulin  $\omega$ -p-Azophenylamyltrimethylammonium Chloride*—The precipitation zones of these dyes were practically identical with those of the corresponding insulin *p*-azobenzyltrimethylammo-

nium chloride preparations. Tests of the dyes with amounts corresponding to 0.5 unit per kilo showed progressive deterioration with an increasing number of groups coupled. When, in order to establish the extent of deterioration, doses corresponding to 1.5 units of insulin per kilo were injected and the results obtained were compared with those obtained with 0.5 unit per kilo of crystalline insulin, 61 per cent deterioration was found. The comparison of the azobenzyltrimethylammonium chloride derivative with the azophenylamyltrimethylammonium chloride derivative showed that there was but little difference between these two insulin dyes.

#### DISCUSSION

The azo dyes of insulin described have not been studied with regard to the manner in which the azo group is coupled to the protein. The preferential place in proteins for the coupling of diazonium salts is, however, the aromatic ring and since the number of coupled groups is relatively small it is reasonable to assume that the azo radicals are linked to the tyrosine and to the histidine nuclei in the insulin. The stability of the dyes at a relatively wide range of hydrogen ion concentrations seems to eliminate the possibility that the azo radicals are linked to amino or hydroxyl groups of the insulin. Reactive azo linkages which would form azo dyes with naphthol in alkaline solution were not present. It seems that an unaltered state of the tyrosine and histidine nuclei is not essential for the physiologic activity of insulin, since the activity was not impaired at all when fifteen strongly acid groups per molecule were coupled through the azo linkages. The fall in potency observed with the derivatives of insulin containing basic groups might in part be due to the low solubility of these compounds at body pH. Some of these derivatives were still found unabsorbed at the place of injection 24 hours later. This was never the case with insulin dyes containing acidic groups.

The size and structure of the substituting group did not seem to affect the activity to any great extent, although there was apparently a greater inactivation with the azophenylamyltrimethylammonium chloride derivative than with the azobenzyltrimethylammonium chloride derivative. The isoelectric

precipitation zones of these two derivatives were indistinguishable from each other. The influence of the strength of the acid group was clearly indicated by the difference found in the physiologic action and physical properties of the derivatives containing sulfonic and of those containing carboxyl groups.

The partial inactivation can hardly have been due to inhomogeneity of the material, since on repeated precipitation the activity was always found to be associated with the coloring matter and the colorless supernatants were inactive.

It is of interest to compare the hypoglycemic activity of the synthetic derivatives of insulin containing basic groups with that of the addition products of insulin with basic proteins such as protamine or globin. Insolubility at neutral pH has been considered responsible for the delay in action of protamine insulin (9). The azo dyes containing basic groups are also practically insoluble at pH 7.3; yet these dyes do not show a delay in action similar to that produced by protamine insulin when injected into fasting rabbits. This fact supports the assumption expressed previously (10) that the delayed action of addition products of insulin is due to their decomposition and liberation of insulin rather than to low solubility.

#### SUMMARY

Azo derivatives of insulin were prepared containing up to fifteen groups per molecule. If the substituting radicals contained anionic groups, the isoelectric precipitation zone was shifted towards the acid side of the isoelectric point of insulin and there was no appreciable loss in potency. Cationic substitution groups caused a shift of the isoelectric precipitation zone towards the alkaline side of the isoelectric point of insulin and some loss in potency.

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# ISOLATION FROM ASTRAGALUS PECTINATUS OF A CRYSTALLINE AMINO ACID COMPLEX CON- TAINING SELENIUM AND SULFUR

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Since the discovery by Robinson (1) of Se in toxic wheat grown on seleniferous soil, one of the most fundamental problems connected with this subject has been to determine in what form the selenium is present in the grain.

Experiments conducted in this laboratory (2, 3) showed that flour prepared from toxic wheat was toxic to rats, and that the toxicity resided in the gluten of the wheat. Gliadin prepared from the gluten proved to be as toxic as the gluten from which it was prepared. These experiments showed definitely that the selenium is combined in some way with the protein of the wheat. It was further found when the gluten was hydrolyzed by boiling with 40 per cent  $H_2SO_4$  for 36 hours that the hydrolytic products possessed the same degree of toxicity as the gluten from which they were prepared, and that there had been no detectable decomposition of the organic selenium compound during the hydrolysis. About the same time Franke, Painter, and associates (4), working independently along similar lines, arrived at essentially the same results.

The results mentioned above suggested that the selenium may be present in the protein as an integral part of one or more amino acids, possibly by replacement of S in cystine or in methionine. With this idea in mind we subjected the hydrolytic products of gluten prepared from toxic wheat to the various procedures commonly employed for the isolation and identification of amino acids from protein hydrolysates, with the object of isolating and

identifying the Se compound. It was found that the dicarboxylic-amino acid fraction contained no Se and that there was little, if any, in the fraction containing the hexone bases, arginine, histidine, and lysine. However, by fractional crystallization of the monoamino acids it was found that most of the Se was concentrated in the leucine fraction, which also contained some valine and phenyl-alanine. By extensive fractional crystallization of this mixture we were not able to isolate a definite Se compound, but we did obtain a small fraction of amino acids (about 200 mg.) which contained about 2 per cent of Se. Because of the difficulties involved in preparing enough of this fraction for further study it was decided to work with a plant material having a much higher content of Se than wheat, which had only 10 to 12 parts per million. Familiarity with the properties of an organic Se compound obtained from this source would be of help later when returning to the study of toxic wheat.

Through the courtesy of Dr. H. G. Byers of the Division of Soil Chemistry and Physics, Bureau of Plant Industry, we were able to obtain a quantity of *Astragalus pectinatus*, a species of vetch which thrives on seleniferous soil in certain areas of the United States. This material (air-dried) contained 1500 to 2000 p.p.m. of Se. It was found that by extracting the finely ground, dry *Astragalus* with hot water about 80 per cent of its Se content could be removed. A great deal of effort was expended in attempting to isolate from the extracts a definite compound containing Se. Direct fractionation, separation by means of various precipitating reagents, and other procedures yielded only sirupy and indefinite products from which nothing could be crystallized or definitely characterized by any criterion of purity. Mercuric acetate gave a precipitate which contained practically all of the Se, but the product was so difficultly soluble that no satisfactory means was found to purify it or to remove the Hg without damage to the organic component. Finally, by adopting a procedure based on the assumption that the organic Se compound had the properties of an amino acid, which we were led to believe from our experience while working with the gluten from toxic wheat, we succeeded in isolating a difficultly soluble, crystalline substance which contains both S and Se, and has all the properties of an amino acid. Its chemical composition and properties agree with those for a combi-

nation of two isomorphous amino acids,  $\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{Se}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$  and  $\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ , in the ratio of 2:1, respectively. These formulae represent a new type of amino acids. The only recorded instance of a compound of this type is that of the unsymmetrical thio ether amino acid which Küster and Irion (5) claimed to have isolated from wool, and to which they assigned a structural formula identical with that given above for the sulfur component of our crystalline substance. Subsequent attempts on their part, however, to isolate the compound were unsuccessful.

Because the quantity of the crystalline product was small and our supply of *Astragalus* had become exhausted, further work had to be suspended for several months until a new supply of *Astragalus* could be obtained. In the meantime we repeated the work of Küster and Irion in the hope that we might isolate the thio ether amino acid which they described. Familiarity gained with its properties would be of value when work was resumed on the analogous S-Se material.

Attempts to isolate this amino acid proved unsuccessful. However, by using an entirely different procedure from that of Küster and Irion, we isolated from wool the closely related symmetrical thio ether, lanthionine,  $\text{HOOC}-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$  (6). Since then, this same amino acid has been isolated from hair, feathers, and lactalbumin.<sup>1</sup> It is of interest to note that the method used for its isolation was essentially the same as that for the final separation of the S-Se crystals from the aqueous extracts of *Astragalus*.

The fact that the properties of lanthionine and the method used for its isolation run so closely parallel with those of the material isolated from *Astragalus* offers additional support to the structure which we have tentatively assigned to it. Both are very difficultly soluble in  $\text{H}_2\text{O}$ . They have high melting points, are readily crystallized from dilute  $\text{NH}_4(\text{OH})$ , and are stable toward strong acids and alkalis.

In addition to the S-Se-containing compound there were isolated from *Astragalus* two other difficultly soluble compounds. It is believed that these represent the two isomorphous components of the S-Se crystals. One, obtained as hexagonal plates, contained

<sup>1</sup> Details of this work will be published later.

27 per cent of Se (29.33 per cent is the theoretical for the Se component,  $C_7H_{14}N_2O_4Se$ ). It gave a weak qualitative test for S, which indicated that it was contaminated with a small amount of the isomorphous mixture. The other compound, considerably more soluble, had a high S content but still contained traces of Se. The quantities available of these substances were too small for further purification. Larger quantities are being prepared for closer study.

The Se-containing amino acid seems to exist in the *Astragalus* in the free state, since at no stage during its isolation was a treatment used that was drastic enough to separate it from a protein or peptide by hydrolysis.

The *Astragalus* used in this study was harvested when it was near or at the bloom stage. The Se amino acid is probably first synthesized in the green plant from the elements and during the later process of metabolism is linked up with other amino acids to form ultimately the plant protein, concentrated largely in the seed. *Astragalus* seeds have been found to contain as high as 3600 p.p.m. of Se.<sup>2</sup> The Se in the seed proteins of wheat and other grains is doubtless accounted for by a similar synthetic process. It may be that the presence of Se modifies the process by which amino acids are normally formed in the plant, so that different types of amino acids are synthesized in order to compensate for the presence of the toxic foreign element.

The results of the work on *Astragalus* have yielded information that not only indicates the type of the Se amino acid present in the gluten of toxic wheat, but also suggests the line of attack to be followed for its isolation. There are a number of points of similarity between the properties of the compound isolated from *Astragalus* and those of the Se compound in the leucine fraction isolated from the hydrolysate of wheat gluten. These properties are also in close agreement with those of lanthionine, and therefore strongly suggest that we are dealing in the three cases with the same type of amino acids.

#### EXPERIMENTAL

Preliminary experiments showed that about 80 per cent of the Se content of the *Astragalus* could be extracted with hot water. Attempts were made to concentrate the Se from the aqueous ex-

<sup>2</sup> Personal communication from Dr. H. G. Byers.

tracts by means of various precipitating reagents, including lead acetate,  $\text{Ba}(\text{OH})_2$ ,  $\text{AgNO}_3$ ,  $\text{HgCl}_2$ , phosphotungstic acid, and mercuric acetate. It was found that nearly all of the Se could be precipitated by mercuric acetate, the reaction being maintained at pH 9 by addition of  $\text{Na}_2\text{CO}_3$ , according to Neuberg and Kerb's procedure for precipitation of amino acids (7). This precipitate was characterized by its remarkable insolubility. When it was suspended in dilute  $\text{HCl}$ , and  $\text{H}_2\text{S}$  was passed into the mixture, only a very small amount of the Se went into solution. The Hg compound was insoluble in cold 20 per cent  $\text{HCl}$ , but when heated it decomposed, yielding a hard, black substance which contained 3 per cent Se, 49 per cent Hg, and 4 per cent N. After many unsuccessful attempts to isolate a crystalline organic Se compound by this type of precipitation this line of attack was abandoned.

When working with the Se-Hg precipitate, we observed that its content of N increased with the Se content. This fact, together with the observations previously made in connection with the fractionation of the amino acids from hydrolyzed gluten from toxic wheat, indicated quite definitely that the Se, at least in part, was combined with amino acids. It was decided, therefore, to attack the problem by taking advantage of the assumption that the Se was present in the form of an amino acid complex. The residue obtained by evaporating the aqueous extract of the *Astragalus* was accordingly taken up in alcoholic  $\text{HCl}$ , and the alcohol was removed by distillation. The sirupy residue was then extracted with absolute alcohol. The alcoholic extract should contain the amino acid hydrochlorides from which the free amino acids could be precipitated by treatment with pyridine in absolute alcohol. After considerable experimentation, the following detailed procedure was adopted.

500 gm. portions of the dried *Astragalus*, freed as far as possible from the stems, were heated on a steam bath with 5 liters of  $\text{H}_2\text{O}$  for 1 hour with frequent stirring. The extract was strained through two layers of cheese-cloth, and the liquid remaining in the residue was removed with the aid of a press. The combined extracts were heated for 30 minutes with 200 gm. of norit. After filtration, the nearly colorless filtrate was evaporated to a thick sirup by distillation *in vacuo*, and the residue was extracted with a warm mixture of 2 liters of absolute alcohol and 100 cc. of con-

centrated HCl. This extraction removed from the sirupy residue the amino acid hydrochlorides, leaving a large quantity of a soluble, gummy carbohydrate which, when dried, had properties resembling those of commercial gum tragacanth. The alcoholic extract was filtered and evaporated to dryness *in vacuo*, and the residue was redissolved in 1.5 liters of warm absolute alcohol. The solution was treated with norit and then filtered. Pyridine was cautiously added to the alcoholic solution until no further precipitation occurred. After the mixture had stood for about 30 minutes, the precipitate was collected in a centrifuge and dissolved in the minimum quantity of hot  $\text{H}_2\text{O}$ . The cooled solution was transferred to a tall cylinder and 3 volumes of pyridine were added. A white, semicrystalline precipitate settled rapidly. After the mixture had stood overnight, the precipitate was removed by filtration. This precipitate, consisting largely of inorganic substance, also included organic material containing Se, S, and N. Analyses showed it to contain 5.6 per cent Se, 3.62 per cent N, and 36.9 per cent ash. A large proportion of the ash was found to consist of P. Ten preparations of this material, each obtained as described from 500 gm. portions of *Astragalus*, were combined and worked up together for purification of the organic Se compound. The combined precipitates were stirred with 400 cc. of hot  $\text{H}_2\text{O}$ . A large part of the material remained undissolved. 100 cc. of concentrated  $\text{NH}_4(\text{OH})$  were then added, which caused the precipitation of additional material. The entire mixture was heated to boiling and allowed to cool before being filtered. The precipitate consisted almost entirely of inorganic material, and contained only a trace of Se. The filtrate was evaporated to a volume of about 100 cc. and a little alcohol was added. When the solution had stood overnight in a refrigerator, the crystalline material which had separated was removed by filtration. By evaporation of the filtrate several additional crops of crystals were successively removed. Addition of alcohol to the final mother liquor yielded a sirupy residue which solidified on standing in the refrigerator. The combined crystalline fractions were dissolved in dilute  $\text{NH}_4(\text{OH})$ , and the filtered solution was slowly evaporated by distillation *in vacuo*. As the  $\text{NH}_3$  was being expelled, an organic Se material separated in the form of rods or long prisms, sometimes admixed with a considerable amount of

asparagine. It was observed that the amount of asparagine which separated at this point depended on the amount of *Astragalus* stems present in the material used at the start. It is, therefore, advisable to use the leaves as free as possible from the stems of the plant. The asparagine, which is much more soluble, can be almost quantitatively removed by extracting the mixture several times with small quantities of hot water. The material remaining undissolved was then dissolved in dilute  $\text{NH}_4(\text{OH})$  and the solution was somewhat concentrated by distillation *in vacuo*. As the  $\text{NH}_3$  was gradually being removed from the solution, the organic Se

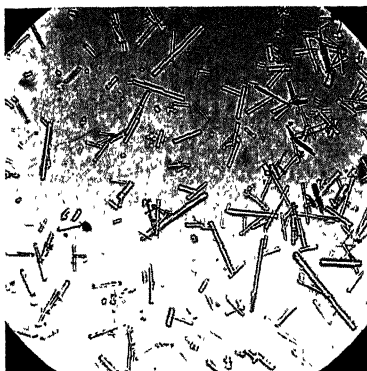


FIG. 1



FIG. 2

FIGS. 1 AND 2. Isomorphic crystals of selenium-amino acid compound from *Astragalus pectinatus*. (Photomicrographs by Mr. G. L. Keenan.)

material separated in the form of apparently homogeneous rod-shaped crystals.

About 0.2 gm. of this material was usually obtained from each 5 kilo lot of the *Astragalus*. Although the quantities obtained from the same amount of *Astragalus* were fairly constant, the yield obtained should not be regarded as quantitative. No endeavor was made to account for all the Se present in the *Astragalus*. The amount contained in the isolated crystalline material represents only a small part of that contained in the plant material. Losses of Se are known to occur. A large amount is removed from the aqueous extract of the *Astragalus* by the norit used for clarification. A considerable amount remains in the



solution after precipitation with pyridine. Other losses occur during subsequent purification.

The first separations of the substance invariably occurred in the form of rods or elongated prisms (Fig. 1), but after several recrystallizations from dilute  $\text{NH}_4(\text{OH})$  in the manner described it separated as thick, lustrous rectangular prisms (Fig. 2). Although the amount of impurity present in the rod form crystals is not enough to affect their percentage composition significantly, the impurity does cause striking effects in the crystallizing habit. Analyses of both crystal forms gave the following percentage composition.<sup>3</sup>

*Analysis*— $\text{C}_{21}\text{H}_{42}\text{N}_6\text{Se}_2\text{O}_{12}\text{S}$

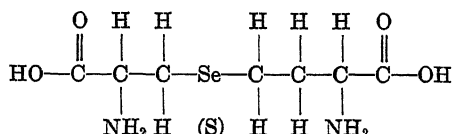
	Calculated.	C 33.16,	H 5.56,	N 11.05,	Se 20.76,	S 4.21
Rod form.	Found.	" 33.38,	" 5.83,	" 10.98,	" 20.62,	" 4.20
Prism	"	" 33.00,	" 5.58,	" 10.98,	" 20.56,	" 4.46

The substance contained no water of crystallization, and it decomposed at  $263\text{--}265^\circ$  (uncorrected). It is very difficultly soluble in  $\text{H}_2\text{O}$ , alcohol, and the common organic solvents, but readily soluble in dilute  $\text{NH}_4(\text{OH})$  and  $\text{HCl}$ . The presence of other constituents of *Astragalus*, such as asparagine, greatly modifies its solubility. Sometimes it will not separate until after the solution has been concentrated to a small volume. The Se compound appears to be very stable. Boiling with 20 per cent  $\text{NaOH}$  caused no apparent decomposition. At no time during this investigation has there been evidence of the presence of any inorganic Se. Qualitative tests showed the presence of N, S, and Se. It is of interest to note that the usual sodium nitroprusside test for S was very indefinite. The presence of Se apparently causes the characteristic reddish violet color of this reaction to disappear almost instantly. Recourse was taken to the specific test for sulfides by the sodium azide spot test (8). This method gave a strongly positive test which removed all doubt as to the presence of S. All the N was found to be in the amino form. Titration of the compound with 0.01 N  $\text{NaOH}$  indicated an equivalent weight of 130.5. It gave a strongly positive test with nin-

<sup>3</sup> Grateful acknowledgment is made to Dr. J. R. Spies and Mr. T. H. Harris of this Bureau for helpful suggestions in connection with the micro-analyses.

hydrin reagent, showing the presence of amino groups in  $\alpha$  position to the carboxyl groups.

The percentage composition of the crystals corresponds to the empirical formula,  $C_{21}H_{42}N_6Se_2SO_{12}$ . Its properties and behavior show definitely that it represents an amino acid complex containing Se and S. By substituting Se for S, the simple formula  $C_7H_{14}N_2O_4Se$  is derived, which suggests that we may be dealing with a combination of two isomorphous compounds,  $C_7H_{14}N_2O_4Se$  and  $C_7H_{14}N_2O_4S$ , in the ratio of 2:1, respectively. Such an assumption is in agreement with the structural formula,



After an 8 month interruption of the work caused by exhaustion of source material, a new supply of *Astragalus pectinatus* was obtained.<sup>4</sup> This, picked at the bloom stage and carefully air-dried, contained 2000 p.p.m. or more of Se. Several preparations of the Se compound described were obtained from this lot of *Astragalus* according to the procedure which has been detailed. The behavior, properties, and composition of the compound were identical in every respect with those obtained from the first lot of material.

During the fractionation of this compound from the inorganic material and asparagine we have recently isolated small quantities of two other crystalline substances. One, a difficultly soluble substance, was separated in the form of hexagonal plates. It contained 27 per cent of Se and a trace of S. The quantity obtained was too small for further examination. Its high Se content, its behavior, and general properties are highly indicative that it was the isomorphous Se component, contaminated with a small amount of the mixture. The calculated Se content of the Se component is 29.33 per cent. Another more soluble crystalline substance having a high S content and a trace of Se was also obtained.

*Simultaneous Determination of Se and S*—Since the available

<sup>4</sup> This supply of *Astragalus* was collected and dried at our request by Dr. H. F. Eppson of the University of Wyoming.

amount of the crystalline organic Se-S compound was small, the quantitative determination of S in the presence of a large amount of Se presented a problem. Inasmuch as the properties of Se and of S are similar in many respects, we were interested in finding out how the Se would behave when the compound was burned in  $O_2$  over a Pt catalyst, according to Pregl's method of combustion in the spiral tube (9). Using a pure Se compound (2-chloro-6-

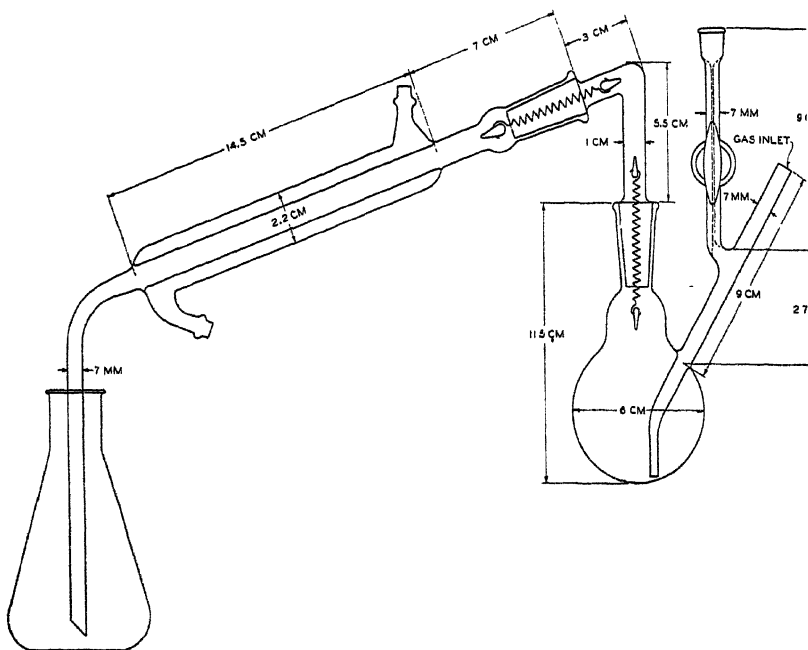


FIG. 3. Apparatus for sulfur and selenium determination

benzyl diselenide) we found that all the Se was oxidized to a mixture of selenite and selenate, and that the Se was partly precipitable by  $Ba(Cl)_2$ . The S was oxidized to  $SO_3$ . On the basis of these observations the following micromethod was worked out for the determination of S and Se in the same sample.

5 to 10 mg. of the organic S-Se compound are burned in the Pregl combustion tube and the combustion products are washed into a distillation apparatus (Fig. 3), which is a modified form of

that described by Robinson, Dudley, Williams, and Byers (10). 5 cc. of Br water (1 cc. of Br in 10 cc. of concentrated HBr) and 10 cc. of concentrated HBr are added. N is slowly bubbled through the reaction mixture in order to keep the liquid in the receiver from being sucked back. Heat is applied gradually and the distillation is continued until only about 5 cc. of liquid remain in the flask. 1 cc. of the Br-HBr solution is poured through the funnel in order to wash out any Se that may adhere to the sides of the condenser. A 10 per cent solution of  $\text{Na}_2\text{SO}_3$  is slowly added to the distillate until the Br color is discharged. The mixture is then warmed until the Se begins to separate. After the mixture has stood overnight, the precipitated Se is collected on an asbestos pad and washed with small portions of  $\text{H}_2\text{O}$ . The Se is dissolved in a small amount of the Br-HBr reagent, and the solution is made up with concentrated HBr to 25 cc. in a volumetric flask. To a 5 cc. aliquot portion a drop of phenol is added in order to remove the free Br. A little KI and starch are then added, and the liberated I is titrated with standard 0.005 N thiosulfate solution.

The residual liquid remaining in the distillation flask, which contains all the S, is washed into a crucible and the S is weighed as  $\text{BaSO}_4$  in the usual way by the Pregl method.

In order to ascertain whether the Br distillation might modify the determination of S by the Pregl method, 10 mg. samples of cystine were burned in the combustion tube and the reaction products subjected to the Br-HBr distillation procedure. All the S was found in the distillation residue in the form of  $\text{SO}_4$ . Several analyses of the Se-S compound, with varying quantities of samples, gave fairly concordant results.

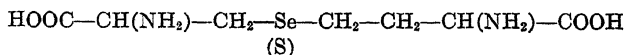
Sample mg.	Sulfur per cent	Selenium per cent
10.250	4.21	20.50
10.798	4.17	20.62
10.536	4.51	20.24
7.689	4.46	20.56

#### SUMMARY

A crystalline organic substance containing Se and S and having all the properties of an amino acid has been isolated from *As-tragalus pectinatus*. The compound is difficultly soluble in  $\text{H}_2\text{O}$ , alcohol, and common organic solvents, but readily soluble in

dilute  $\text{NH}_4(\text{OH})$  and  $\text{HCl}$ . The Se substance is very stable. Boiling with 20 per cent  $\text{NaOH}$  causes no apparent decomposition. The percentage composition corresponds to a mixture of two isomorphous compounds,  $\text{C}_7\text{H}_{14}\text{N}_2\text{O}_4\text{Se}$  and  $\text{C}_7\text{H}_{14}\text{N}_2\text{O}_4\text{S}$ , in the ratio of 2:1, respectively.

The following structure has been tentatively assigned to each component.



It is believed that the Se in toxic wheat and other grains is combined in the protein as an amino acid having a similar structure.

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## OBSERVATIONS ON CARBONIC ANHYDRASE

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Carbonic anhydrase is an enzyme which catalyzes both phases of the reversible reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$ . The presence of this enzyme in mammalian red blood corpuscles was first demonstrated by Meldrum and Roughton (1) and independently by Stadie and O'Brien (2). Since the early work of these investigators, many contributions have appeared on methods of estimating the biological activity and on the purification of the enzyme. In 1933, Brinkman *et al.* (3) described a glass boat apparatus by which the activity of the enzyme in catalyzing the reaction  $\text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O}$  could be measured manometrically. Later Philpot and Philpot (4) described a colorimetric assay method. Meldrum and Roughton (5) established the unit of activity of the enzyme and further elaborated methods of purification. Their purest product contained 1730 units per mg. Mann and Keilin (6) reported that preparations of carbonic anhydrase possessing a high degree of activity had a relatively high zinc content. In a later communication these authors (7) concluded that carbonic anhydrase is a Zn-protein compound. The work of Hove, Elvehjem, and Hart (8) further confirmed the Zn-protein nature of this enzyme. Kiese and Hastings (9) published a method for preparing the enzyme and reported a product which was several times more active than that obtained by Meldrum and Roughton. Recently, Keilin and Mann (10) described two methods for purifying carbonic anhydrase. The resultant product obtained by each process of purification contained approximately 2220 units per mg. From this and other considerations they conclude that their preparations are "pure or almost pure."

\* Holder of a fellowship of the Asociacion Argentina para el Progreso de las Ciencias.

Further, they are unwilling to accept the estimations of very great potency, reported by Kiese and Hastings, and suggest that these were "probably due to the low temperature (5° instead of 15°) at which their estimations were carried out."

Since the first report by Keilin and Mann that carbonic anhydrase is a Zn-protein, we have been greatly interested in this enzyme. The fact that the enzyme occurs in pancreas (11), the only gland from which another highly active Zn-protein (12, 13), insulin in crystalline form, can be prepared, stimulated us to conduct certain experiments. Accordingly, it became necessary to prepare a quantity of the purified enzyme. Many of the steps used in the preparation of the enzyme which are reported in the present paper have been used by other investigators. In our method of purification, practically all operations have been conducted at room temperature and, as far as possible, the use of such solvents as alcohol or acetone has been avoided, since even 50 per cent concentrations of these substances readily inactivate the enzyme.

The purified carbonic anhydrase which we isolated proved to be very unstable in dilute solutions. Our colleague, P. J. Moloney (14), who had been faced with the problem of stabilizing dilute solutions of diphtheria toxin, found the addition of gelatin or peptone greatly enhanced its stability. We therefore decided to determine whether or not these substances might also be used as stabilizing agents for carbonic anhydrase. When they were used with this enzyme, not only was a stable product obtained but a greatly increased activity was noted. Since the methods of purification and testing may be factors which enter into the results which we are about to report, we are describing them in some detail. In addition to the experiments on stability, others are recorded in order better to understand the reason for this marked increase in the activity of the enzyme.

#### EXPERIMENTAL

*Method of Assay*—The catalytic activity of carbonic anhydrase was measured by the manometric method. A glass boat apparatus similar to that described by Brinkman *et al.* was used for the reaction chamber.

In this method 2.0 cc. of a stock phosphate buffer solution were

placed in one side of the boat. The solution was prepared by mixing equal volumes of 0.2 M  $\text{Na}_2\text{HPO}_4$  and 0.2 M  $\text{KH}_2\text{PO}_4$ . In the other side of the boat were placed 2.0 cc. of sodium bicarbonate solution made up according to the method of Hodgson (0.186 M  $\text{NaHCO}_3$  and 0.038 M  $\text{NaOH}$ ) (15). The boat was then immersed in a water bath and connected by means of a rubber stopper and rubber tubing to the manometer. Water was added to the bath until both the boat and stopper were completely immersed. The temperature of the water was then adjusted and maintained at 15°. When the temperature of the boat had come into equilibrium with that of the bath, the boat was violently shaken in a horizontal position by means of a mechanical shaker. The rate of shaking was 355 per minute. When shaking commenced, the solutions in the glass chamber became thoroughly mixed,  $\text{CO}_2$  was evolved, and the amount was recorded on the manometer. When the water of the manometer had risen 1.0 cm., the time was noted by starting a stop-watch graduated in 0.01 second. Shaking was continued until the water of the manometer had risen a further 3.0 cm. The watch was then stopped, and the time recorded. The distance between the water levels in the manometer at the time of reading was therefore 8.0 cm. The time required when freshly prepared bicarbonate solutions were used in the reaction chamber was generally about 45 seconds. After the bicarbonate solution has been allowed to stand for a few weeks, the time may increase to 50 seconds and even longer. In our work, when the reaction time was more than 50 seconds, the bicarbonate solution was discarded and a fresh one prepared.

In the catalyzed reaction 0.5 cc. of a suitably diluted solution of carbonic anhydrase was added to the side of the boat containing the phosphate buffer. The temperature and the shaking were the same as in the control experiment. In our work, the enzyme was so diluted that the time necessary for the water to rise in the manometer the same distance as in the control experiment was from 12 to 25 seconds. For each determination of enzyme activity, a control experiment was made with the reagents containing no enzyme. All determinations of blank or catalyzed systems, except only those of the first line of Table I, were made in duplicate.



We have adopted the Meldrum and Roughton (5) formula for estimating the enzyme unitage, the unit being the amount of enzyme which, when dissolved in 4 cc. of the phosphate-bicarbonate mixture gives a value  $(R - R_0)/R_0$  equal to 1 at 15°, where  $R_0$  and  $R$  are respectively the rates of reaction in the absence and in the presence of the catalyst.

We have found it important to use only sterile glassware.

#### *Purification of Carbonic Anhydrase*

*Washing of Erythrocytes and Extraction with Alcohol and Chloroform*—The red corpuscles of 18 liters of defibrinated ox blood, after the removal of 1 cc. for the estimation of carbonic anhydrase activity, were separated from the serum by centrifugation. The erythrocytes were washed and centrifuged three successive times with 0.9 per cent NaCl. The amount of saline used was 21.5 liters. The erythrocytes were plasmolyzed, 100 cc. of distilled water being added to the cells obtained from each 250 cc. of the defibrinated blood. 150 cc. quantities of the hemolyzed blood were placed in each of a series of centrifuge tubes. After they had stood in a refrigerator for 3 hours, 25 cc. of chilled alcohol were added to each tube and the tubes well shaken. They were then placed at room temperature and 45 cc. of chloroform added to each. Each tube was vigorously shaken on the addition of the chloroform (16). After standing 2 hours the tubes were centrifuged and the supernatant yellow liquid containing most of the enzyme decanted. The volume of liquid was 11.8 liters.

Total carbonic anhydrase activity

In original blood . . . . .	71,000,000 units
After alcohol and chloroform ..	50,654,000 "

*Precipitation of Enzyme with Lead Acetate and Ammonium Sulfate*—200 cc. quantities of the enzyme preparation were measured into a series of centrifuge tubes. To each tube were added 15 cc. of a saturated solution of basic lead acetate and after shaking they were allowed to stand 0.5 hour. They were then centrifuged and the supernatant liquid discarded. The precipitate in each tube was thoroughly mixed with 100 cc. of 0.2 M  $K_2HPO_4$ . After standing 0.5 hour the tubes were again centrifuged and the supernatant phosphate liquids combined. The volume of solution

was 6200 cc. and the pH was 10.5. The reaction of the solution was adjusted to pH 7.7 by adding approximately 250 cc. of N acetic acid.

To the enzyme solution, ammonium sulfate was added to saturation (70 gm. per 100 cc.). The precipitate which formed was allowed to stand overnight and was then removed by filtration. The precipitate containing the enzyme was dissolved in 400 cc. of water, and dialyzed for 15 hours. The volume after dialysis was 950 cc.

Total carbonic anhydrase activity

After lead treatment .....	35,862,000 units
After ammonium sulfate and dialysis .	34,050,000 "

*Further Purification with Alcohol and Chloroform and with Ammonium Sulfate*—This enzyme solution now had a slightly reddish color and was treated with 175 cc. of alcohol and 300 cc. of chloroform. The mixture was well shaken and a reddish precipitate removed by centrifugation. The clear alcoholic solution of the enzyme was dialyzed for 15 hours. The volume was then 1260 cc.

The enzyme was again precipitated with ammonium sulfate (70 gm. per 100 cc.). After standing overnight the precipitate was removed by filtration and dissolved in 130 cc. of water. This solution was dialyzed for 15 hours and its volume was 275 cc.

Carbonic anhydrase

After alcohol, chloroform, and dialysis	34,000,000 units
After ammonium sulfate and dialysis .	34,000,000 "

*Treatment with Lead Acetate*—When a small amount of basic lead acetate is added to the enzyme solution, a flocculent precipitate appears which contains no enzyme activity. If too much lead is added, however, there is a tendency for a part of the precipitate to redissolve. Hence, care must be exercised in order to remove the maximum amount of impurities. In our preparation, 5 cc. of a saturated solution of basic lead acetate were added to the 275 cc. of enzyme solution. The precipitate which formed was removed by centrifugation and the excess lead in the supernatant solution precipitated by adding a small amount of  $K_2HPO_4$ . The insoluble lead phosphate was removed by centrifugation and

the supernatant solution dialyzed for 15 hours. After dialysis the volume was 280 cc.

Carbonic anhydrase after lead acetate treatment and dialysis, .  
33,000,000 units

*Purification with Aluminum C $\gamma$  Gel*—The purified enzyme solution now was bluish in color owing to the presence of hemocuprein. This impurity can be removed completely by aluminum C $\gamma$  gel (17). To the enzyme solution, 15 cc. of aluminum C $\gamma$  gel, containing 16 mg. of dry substance per cc., were added. The reaction of the mixture was pH 7.2. After standing several hours, another similar quantity of aluminum gel was added, and again the suspension was allowed to stand. This procedure was repeated until a total volume of 125 cc. of aluminum C $\gamma$  gel had been added. The gel was then removed by centrifugation, practically all the enzyme being in the supernatant solution. This solution contained less than 1  $\gamma$  of copper per cc. The volume of enzyme solution was 340 cc. To this solution was added 0.5 gm. of K<sub>2</sub>HPO<sub>4</sub>. A very faint turbidity of aluminum phosphate formed. After standing overnight it was removed by centrifugation and the enzyme solution was dialyzed first against tap water, and then distilled water.

Carbonic anhydrase after treatment with aluminum C $\gamma$ ,  
30,800,000 units

The aqueous solution was dried in a Desivac machine which is used for drying human blood sera. The dried enzyme preparation was snow-white and had a fluffy nature. Weighed amounts of this enzyme were dissolved in water and used for numerous experiments.

In the course of experiments which will form the subject of a later communication, difficulty was encountered in obtaining results which were consistent with the experiments in progress. It was noted that potency tests on a freshly diluted sample of the enzyme always gave a higher value than those obtained after the diluted solutions had been allowed to stand for an hour or even less. Accordingly, it was decided to carry out a stability experiment at 20°. In this experiment a freshly weighed sample of purified carbonic anhydrase was diluted with water so that

each cc. contained 2.03 mg. of carbonic anhydrase. 1.0 cc. of this solution was diluted with 3200 cc. of water and a potency test immediately made. The dilute enzyme solution was allowed to stand at 20° and further potency tests made during a 24 hour period. The results of this experiment are shown in Table I.

Since dilute solutions of our preparation of carbonic anhydrase were very unstable (Table I), it was decided to carry out potency estimations on dilute solutions of the enzyme containing known amounts of a substance which might act as a stabilizer. In Table II are the results of such an experiment. In this experiment, solutions of peptone (Witte) of various concentrations were prepared. 1.0 cc. of the stock enzyme solution (2.03 mg. of enzyme) was diluted to 3200 cc. with each of the various peptone solutions.

TABLE I  
*Stability of Carbonic Anhydrase in Water at 20°*

Time standing before test	Carbonic anhydrase activity
	<i>units per mg.</i>
10 min.	4100
0.5 hr.	3030
1.5 hrs.	2130
2.5 "	1480
4.0 "	1290
7.0 "	730
24.0 "	0

The enzyme activity was determined immediately after dilution, and after standing for 20 hours at 20°. The results of these experiments are shown in Table II.

From the results of the experiments (Table II) in which carbonic anhydrase is diluted with various concentrations of peptone, certain facts are obvious. First, the presence of peptone even in very dilute concentrations greatly increases the stability of the enzyme. Secondly, maximum activity of the enzyme is obtained when the carbonic anhydrase is diluted with a 0.05 per cent solution of peptone. This activity is almost 4 times that reported for the pure product of Keilin and Mann. Lastly, it will be noted that a stable product is obtained at much lower concentrations of peptone than is required to secure maximum activity. This

fact might strongly suggest that the peptone solutions not only act as stabilizers but also contain some substance which activates the carbonic anhydrase. With this in mind, another series of experiments was carried out, the results of which are shown in Table III.

In Experiments 1 and 2, Table III, attempts were made to determine whether the peptone contained a heat-labile substance that was acting as an activator. A peptone solution of 0.05 per cent was prepared. One-half of this solution was boiled for 0.5 hour and then allowed to cool. Carbonic anhydrase was then

TABLE II  
*Activity of Carbonic Anhydrase in Various Concentrations of Peptone Solutions*

Experiment No.	Peptone concentration*	Tested immediately	Tested after 20 hrs. at 20°
	<i>per cent</i>	<i>units per mg.</i>	<i>units per mg.</i>
1	0.00000	3980	240
2	0.00025	3960	1640
3	0.0010	4820	3930
4	0.0017	5680	5060
5	0.0033	5900	5900
6	0.0065	6580	6580
7	0.013	7470	7420
8	0.025	7700	7930
9	0.050	8260	8100
10	0.10	7980	7920
11	0.20	7620	7740

\* On account of the addition of the other components, the concentration of peptone in the reaction chamber was, of course, one-ninth of these values.

added to each of the heated and unheated peptone solutions, so that each contained 2.03 mg. of enzyme per 3200 cc. of solution. The enzyme activity of these solutions was tested immediately and after 20 hours at 20°.

It was thought that perhaps the instability of the enzyme might be due entirely to the vigorous shaking in the boat during the test. Accordingly, in Experiments 3 and 4, a test was made on a solution of carbonic anhydrase dissolved in distilled water (2.03 mg. in 3200 cc.). This solution was then allowed to stand for 20 hours at 20° and again tested. To the remainder of the solution, peptone was added to 0.05 per cent concentration and a test made.

Experiments 5 and 6 were planned to find out whether the peptone contained dialyzable substances which acted as activators or stabilizers. A peptone solution was prepared of 0.05 per cent concentration. One-half of this solution was dialyzed for 24 hours. Carbonic anhydrase was then added to the dialyzed and non-dialyzed peptone solutions so that each contained 2.03 mg. of enzyme per 3200 cc. of solution. The enzyme activity of these solutions was tested immediately and after 20 hours at 20°.

TABLE III

*Miscellaneous Experiments with Carbonic Anhydrase*

The carbonic anhydrase activity is measured in units per mg.

Ex- peri- ment No.		Carbonic anhy- drase activity	
		Tested im- medi- ately	Tested after 20 hrs. at 20°
1	Carbonic anhydrase diluted with 0.05% peptone	7800	7800
2	" " " " 0.05% " that had been boiled for 0.5 hr.	7800	7800
3	Carbonic anhydrase diluted with distilled water	4100	0
4	After standing 20 hrs., peptone to 0.05% concentra- tion added to solution of (3)		240
5	Carbonic anhydrase diluted with 0.05% peptone	7820	8040
6	" " " " 0.05% " that had been dialyzed for 24 hrs.	8450	8450
7	Carbonic anhydrase diluted with water	4100	450
8	" " " " " containing ash of 2 mg. carbonic anhydrase	4160	300
9	Carbonic anhydrase diluted with water	4100	450
10	" " " " " containing ash of 100 mg. peptone	4190	570
11	Carbonic anhydrase diluted with 0.0033% peptone	5700	5400
12	After standing 20 hrs., peptone to 0.05% concentra- tion added to solution of (11)		5700

In Experiments 7 and 8, an attempt was made to ascertain whether the ash of carbonic anhydrase contained an activator. 2.03 mg. of carbonic anhydrase were ashed and the ash dissolved in distilled water. Suitable dilutions of the enzyme were then made (2.03 mg. in 3200 cc.) with distilled water and with the water containing the ash. These solutions were tested for activity immediately after dilution and after standing 20 hours at 20°.

Experiments 9 and 10 were designed to determine whether the

ash of peptone contained an activator for carbonic anhydrase. 0.1 gm. of peptone was ashed and the ash dissolved in 200 cc. of distilled water. Carbonic anhydrase was then added to distilled water and to the distilled water containing the peptone ash, so that each contained 2.03 mg. of enzyme per 3200 cc. of solution. Both solutions were tested for enzyme activity immediately after dilution and after standing 20 hours at 20°.

Since carbonic anhydrase is stable in a 0.0033 per cent solution of peptone (Table II), it seemed important to find out whether such a solution, after standing 20 hours and then having the peptone concentration increased to 0.05 per cent, would exhibit its maximum activity. The results of this experiment are shown in Experiments 11 and 12. In these experiments, 1.0 cc. of carbonic anhydrase (2.03 mg.) was diluted to 3200 cc. with the peptone solution (0.0033 per cent). This solution was tested immediately and after it had stood 20 hours at 20°. To the remaining solution, peptone to 0.05 per cent concentration was added and the activity of the enzyme again determined.

It seemed important to determine the activity and stability of carbonic anhydrase in the presence of substances other than peptone. Accordingly, a series of experiments was undertaken in which various substances were diluted with distilled water to 0.05 per cent concentration. Carbonic anhydrase was then added to each of these solutions, so that each contained 2.03 mg. of the enzyme per 3200 cc. of solution. Potency tests were made on the solutions immediately after dilution with the enzyme and on the solutions after they had stood for 20 hours at 20°. Of the substances used, only one can be considered as a pure substance; namely, insulin. The insulin was first crystallized and then practically all the zinc removed by trichloroacetic acid and alcohol and ether. After the insulin was dissolved, the reaction was adjusted to pH 6.8.

#### DISCUSSION

A method for preparing very active preparations of carbonic anhydrase is described. This method resulted in the recovery of over 40 per cent of the carbonic anhydrase contained in defibrinated blood. In testing the various fractions during the purification, dilutions for assay purposes were always made with a solu-

tion of 0.05 per cent peptone in order to insure a constant potency over a reasonable length of time.

From the results of a stability experiment (Table I) it will be noted that the enzyme became completely inactivated on standing at room temperature in a dilute peptone-free solution in 24 hours. The results of experiments in which various concentrations of peptone were used to dilute the enzyme (Table II) indicate that not only does peptone increase the stability of dilute solutions of the enzyme but also gives activity values for carbonic anhydrase which are approximately 4 times those that have been reported for the pure enzyme. It will also be noted that the concentration of peptone required to demonstrate a maximum activity is greater than that necessary to insure stability. This fact would suggest that not only is the peptone a stabilizer for the enzyme but also that it contains an activator which is necessary for maximum enzyme activity. From the results of Table III it will be seen that boiling the peptone solution did not interfere with its ability to cause increased enzyme activity (Experiments 1 and 2). Thus the activator, if such exists, is heat-stable and probably not of the nature of a coenzyme. It would appear that most of the enzyme activity is lost during standing in a dilute solution rather than during the shaking in the glass boat (Experiments 3, 4, 11, and 12). From the results (Experiments 5 and 6) it would appear that the activator, if present, is not dialyzable. Further, neither the ash of carbonic anhydrase nor the ash of peptone contains substances which materially stabilize or activate carbonic anhydrase (Experiments 7 to 10).

From the results of experiments (Table IV) it is evident that various substances can be used to improve the stability of the enzyme. The enzyme exhibits approximately the same maximum activity in the presence of any one of peptone, insulin, and blood serum. The other substances probably would have produced a greater enzyme activity had a greater concentration of them been used. This is known to be true in the case of gelatin. In carrying out these tests, saponin was unsatisfactory, since it caused excessive frothing, giving a control value which was unreasonably high. The control for insulin was slightly higher than normal but we do not believe it interfered with the accuracy of the results. Solutions of none of the other substances gave a control that dif-



ferred from that of experiments in which only water was used. The experiment with insulin, a pure protein, would indicate that the increased activity observed when various substances are added to dilute solutions of carbonic anhydrase is due to a stabilizing effect rather than to an activator. If insulin is an activator, then activation must be due to the protein itself. Such a possibility, however, is unlikely, since both peptone and blood serum when added to the enzyme give activity values which are comparable with that caused by the addition of the insulin protein. Moreover, the amount of insulin used in these experiments is much too great for it to be considered as an activator for the enzyme. In other

TABLE IV  
*Stability of Carbonic Anhydrase in Water Containing 0.05 Per Cent  
Concentration of Various Substances*

Substance added to water	Carbonic anhydrase tested immediately	Activity tested after 24 hrs.
	<i>units per mg.</i>	<i>units per mg.</i>
Peptone . . . . .	7800	7800
Gelatin . . . . .	5360	4200
Insulin . . . . .	8130	7960
Saponin . . . . .	5330	3810
Blood serum (human)	7660	8240
Protamine. . . . .	6160	5970
Egg albumin*	4500	1600
Edestin* . . . . .	5830	5170
Casein* . . . . .	5000	3760

\* These substances did not completely dissolve in water.

experiments in which only very small amounts of insulin were used with the enzyme no marked increase in enzyme activity was observed. The fact that both the enzyme and insulin have an affinity for zinc may be a factor entering into the results which have been obtained. The insulin which was used contained a small amount of sodium chloride. This substance, however, in the amounts contained in the insulin, had no effect on the stability of dilute solutions of the enzyme.

As this paper was being sent to press, our attention was drawn to an abstract of a paper by Leiner and Leiner (18). The original paper is not available in our country at this time. These authors

found that dialysis reduced the activity of carbonic anhydrase by 50 per cent, and that the dialysate contains an activator. They also reported that serum and many organs contain this activator and that purified preparations of enzyme preparations are activated at a higher rate than are crude preparations of the enzyme. Our results confirm the findings of these authors in one respect; namely, that serum proteins increase the potency values of purified carbonic anhydrase. Our results do not indicate that carbonic anhydrase contains a dialyzable activator which is heat-, acid-, and alkali-resistant, nor that any activity is lost during dialysis, as claimed by Leiner and Leiner.

#### SUMMARY

A method of preparing very active carbonic anhydrase from defibrinated blood is described. This enzyme in purified form is very unstable in dilute solutions. Many substances when added to such solutions stabilize the enzyme and also give greatly increased activity values, about 4 times the activity previously reported for the pure enzyme. Attempts to identify this increased activity with any inorganic or dialyzable substance have failed. The fact that insulin, a pure protein, behaved in a manner similar to peptone or blood serum suggests that the increased activity of carbonic anhydrase in dilute solutions is due to a stabilizing effect rather than to the presence of an activator.

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## A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF NICOTINIC ACID

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Recognition of the biochemical importance of nicotinic acid and its amide has made methods for their determination under a variety of conditions of great interest.

Numerous procedures have been recommended for the assay of nicotinic acid. They have as their bases (a) color reactions produced by interaction of pyridine derivatives and cyanogen bromide with an aromatic amine (1-6), (b) the color reaction produced with pyridine derivatives and 2,4-dinitrochlorobenzene (7, 8), (c) a method curative of blacktongue in dogs (9), (d) microbiological methods based on its essential nature for microorganisms (10).

Compounds possessing no anti-blacktongue or antipellagric activity such as pyridine derivatives (especially trigonelline under certain conditions (11)) and other compounds of unknown nature interfere in chemical methods for determining nicotinic acid. Especially is this so in the case of grains and similar materials (12). A method depending on biological specificity rather than chemical reactivity thus has a unique advantage in the study of physiologically active compounds.

Assay with higher animals is, however, inaccurate, time-consuming, and expensive. Microbiological methods so far presented are unsatisfactory on one or more of the following counts: (a) they employ pathogenic organisms; (b) the basal media used are not adequately supplied with compounds which supplement the effect of added nicotinic acid, and hence the methods are non-specific; (c) the quantitative response to nicotinic acid may differ greatly from that to nicotinamide (13); (d) the necessary use of turbidity as the means for following growth introduces limitations when the method is applied to colored or turbid extracts.

Many of the lactic acid bacteria are characterized by a requirement for nicotinic acid as an essential growth factor (14). Members of this genus have previously been employed for the microbiological assay of riboflavin (15) and pantothenic acid (16). These methods have been shown to possess certain advantages in the assay of naturally occurring products because of their specificity, accuracy, rapidity, and sensitivity. The following procedure has been found suitable for the determination of nicotinic acid in a wide variety of materials. It has a sensitivity 20 to 100 times that possible with existing chemical methods, and meets satisfactorily the objections raised above to previous microbiological methods.

#### EXPERIMENTAL

*Organism*—The organism used is *Lactobacillus arabinosus* 17-5.<sup>1</sup> Stab cultures are carried in yeast extract-glucose agar (1 per cent glucose, 1 per cent yeast extract, 1.5 per cent agar). These stabs are prepared from previous stock cultures at monthly intervals. After transfer, cultures are incubated at 30° for 24 to 48 hours, then held in the refrigerator. Inoculum for assay tubes is prepared by transfer from the stock culture to a sterile tube of the basal medium (Table I) to which 0.1  $\gamma$  per cc. of nicotinic acid has been added. The inoculum is incubated at 30° for 18 to 36 hours before use.

The constituents of the medium are prepared as follows:

*Acid-Hydrolyzed Casein*—50 gm. of vitamin-free casein (Labco) are hydrolyzed with 250 cc. of 25 per cent sulfuric acid. Ordinary technical casein (and some "vitamin-free" caseins) contains considerable amounts of nicotinic acid. The mixture is autoclaved for 10 hours at 15 pounds pressure. The sulfuric acid is removed with barium hydroxide. Any excess barium ion is carefully removed with a minimum amount of sulfuric acid, and the solution is adjusted by dilution or evaporation to contain 100 mg. of dry matter per cc. It is preserved under toluene. Traces of nicotinic acid (and other vitamins) can be more completely removed from the casein hydrolysate by stirring the above solution at pH 3.0 with 10 mg. per cc. of active charcoal, and filtering, but this is not recommended as a general procedure.

<sup>1</sup> Cultures of this organism may be secured from the American Type Culture Collection, Georgetown University Medical School, Washington, D. C., where it is listed as No. 8014.

*Cystine*—A solution of cystine hydrochloride containing 1 mg. of cystine per cc. is prepared by suspending cystine in water and adding the least possible amount of hydrochloric acid to effect solution and then diluting to the required volume with water. It is kept under toluene.

*Adenine, Guanine, and Uracil*—A solution is prepared containing 1 mg. per cc. of each of these constituents. Solution is effected by prolonged heating in the presence of a few drops of hydrochloric acid. It is stored in the refrigerator and renewed at frequent intervals.

*Thiamine, Calcium Pantothenate, and Vitamin B<sub>6</sub>*—Stock solutions are prepared containing 100  $\gamma$  per cc. dissolved in distilled

TABLE I  
*Basal Medium*

Acid-hydrolyzed casein.....	0.5%
Tryptophane.....	0.01%
Cystine.....	0.01%
Glucose.....	1.0%
Sodium acetate.....	0.6%
Adenine.....	10 p.p.m.
Guanine.....	10 "
Uracil.....	10 "
Thiamine.....	0.1 "
Calcium pantothenate.....	0.1 "
Vitamin B <sub>6</sub> .....	0.1 "
Riboflavin.....	0.2 "
Biotin (concentrate) $\approx$ .....	0.4 part per billion pure biotin
Inorganic salts.....	See text

water. They are stored in the refrigerator and renewed at frequent intervals.

*Riboflavin*—A solution of riboflavin containing 100  $\gamma$  per cc. is prepared in 0.02 N acetic acid and kept in the refrigerator. Unnecessary exposure to light should be avoided and a fresh solution prepared at frequent intervals.

*Biotin*—Any biotin concentrate which is free from significant amounts of nicotinic acid may be used. Present commercial concentrates have not been tested in this regard. Suitable concentrates can be obtained by following Kögl and Tönnis' original procedure (17) with fresh egg yolk through the first charcoal adsorption. We have found the following procedure, starting

with egg yolk, to be convenient. Forty-eight eggs are boiled for 15 minutes; the yolks are removed, mashed, and then extracted twice by steaming in the autoclave with two 1000 cc. portions of water. The combined filtrates are concentrated *in vacuo* to 50 cc. 50 cc. of acetone are added, the precipitate centrifuged out, and the acetone removed by evaporation to 50 cc. 250 cc. of methanol are now added, and the precipitate discarded. The methanol is removed by evaporation. The extract is diluted to 100 cc., adjusted to pH 5.5, and extracted with two 300 cc. portions of amyl alcohol. The alcohol extract is discarded. The aqueous phase is adjusted to pH 3.0 with sulfuric acid and extracted four times with 300 cc. portions of amyl alcohol. The aqueous phase is discarded. The biotin is extracted from the amyl alcohol by shaking with portions of  $\text{Ba}(\text{OH})_2$  solution until the water layer remains alkaline. Barium ion is removed from the extract with sulfuric acid, and the extract concentrated to 15 cc. 0.6 cc. of concentrated sulfuric acid is now added, and a 50 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid is added to complete precipitation. The precipitate is removed, washed once with 5 per cent sulfuric acid, and then decomposed in the usual manner with barium hydroxide. Excess barium ion is removed with sulfuric acid. The final concentrate contains from 400 to 1000  $\gamma$  of biotin per gm. The concentrate can be standardized by the method of Snell *et al.* (18), and the recommended amount added to the medium. Portions of the concentrate are diluted to contain 0.2  $\gamma$  of biotin per cc. for immediate use. In the absence of suitable biotin standards the amount to be added can be determined with the above medium and organism by adding an excess (1 to 2  $\gamma$ ) of nicotinic acid per tube of medium and then determining the amount of concentrate necessary to obtain maximum growth (as determined by acid titration after 3 days incubation). About 4 times this amount are then added per tube of medium for the routine determination of nicotinic acid.

*Inorganic Salts*—Solution A contains 25 gm. of potassium monohydrogen phosphate and 25 gm. of potassium dihydrogen phosphate in 250 cc. of water.

Solution B contains 10 gm. of magnesium sulfate heptahydrate, 0.5 gm. of sodium chloride, 0.5 gm. of ferrous sulfate heptahydrate, and 0.5 gm. of manganese sulfate tetrahydrate dissolved in 250 cc.

of water. Salts precipitate from Solution B when it stands in air; it need be renewed only when a uniform suspension can no longer be secured by shaking.

### Procedure

Assays are carried out in 6 inch bacteriological test-tubes. These are conveniently supported by a wire or metal rack which may be autoclaved. If, for example, ten assay tubes are to be prepared from stock solutions as described above, the following amounts should be used: 5 cc. of casein hydrolysate solution, 10 mg. of tryptophane, 10 cc. of cystine hydrochloride solution,

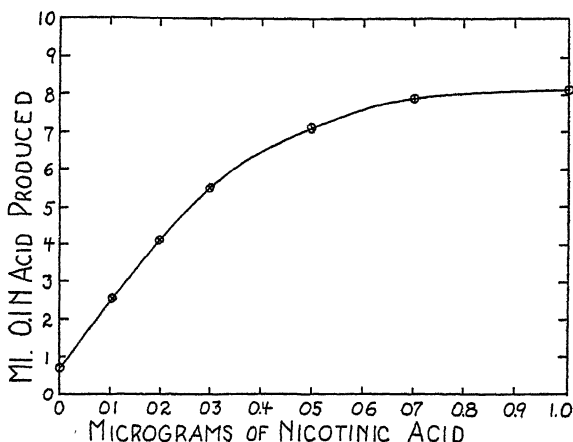


FIG. 1. Response of *Lactobacillus arabinosus* 17-5 to added nicotinic acid.

1 gm. of glucose, 0.6 gm. of sodium acetate, 1 cc. of adenine, guanine, and uracil solution, 0.5 cc. of inorganic salts, Solutions A and B, 0.1 cc. of thiamine, pantothenic acid, and vitamin B<sub>6</sub> solutions, and 0.2 cc. of riboflavin and biotin solutions.

The mixture is adjusted to pH 6.6 to 6.8 and diluted to 50 cc. This is double the concentration of the final medium. The standard nicotinic acid solution and solutions for analysis are added to the tubes. Duplicate tubes containing 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0  $\gamma$  of nicotinic acid are set up for the standard curve (Fig. 1). The samples for assay are set up at increasing levels of concentration estimated to contain between 0.02 and 0.4  $\gamma$  of nicotinic acid.



Volumes up to 5 cc. may be used. The contents of all the tubes are then diluted if necessary to 5 cc. with water; then 5 cc. of the basal medium prepared as described above are pipetted into each tube. The tubes are plugged with cotton and sterilized in the autoclave at 15 pounds steam pressure for 15 minutes. After cooling to room temperature, they are ready for inoculation.

The cells from a 24 hour culture of inoculum grown as described previously are centrifuged out aseptically and resuspended in 0.9 per cent NaCl solution. 1 drop of the resulting suspension is added to each assay tube. Aseptic precautions must be observed. The tubes are incubated at 30° for a growth period of approximately 72 hours.

*Measurement of Response to Nicotinic Acid*—Titration of the lactic acid produced has been employed as a measure of the response to nicotinic acid. Brom-thymol blue is a satisfactory indicator. Titrations are reproducible to 0.1 cc. of 0.1 N sodium hydroxide.

### Results

The response to added nicotinic acid is shown in Fig. 1. A standard curve similar to Fig. 1 must be obtained with each set of assays. The nicotinic acid content of each "unknown" tube is then read from the standard curve. From these values the nicotinic acid content of the sample is calculated. The average of values obtained at several levels on the curve within the assay limits is used (Table II). Values near the extreme lower portion of the curve or the upper flat region are not used for assay purposes.

Results of several hundred assays on a variety of materials have shown that the average value calculated from several levels is reliable even though the sample shows a trend upward or downward at increasing assay levels (*cf.* liver, Table II). Recoveries of added nicotinic acid may vary in this same manner and may be considerably in error if based on a single point, but they are entirely satisfactory when the average of several points along the curve is taken (Table III).

Nicotinic acid and nicotinamide have equal activities for this organism. A sample of cozymase<sup>2</sup> was assayed and found to

<sup>2</sup> We wish to thank Dr. A. E. Axelrod for a sample of pure cozymase, and Dr. T. D. Spies for a sample of nicotinuric acid.

contain 17 per cent nicotinamide (theory 18 per cent). Trigonelline is inactive; nicotinuric acid shows the same activity as its theoretical equivalent of nicotinic acid (found 74 per cent; calculated 68.3 per cent (Table III)).

*Preparation of Sample for Analysis*—The pretreatment of the sample for analysis will vary somewhat with the material to be assayed.

TABLE II  
*Nicotinic Acid Content of Materials at Varying Assay Levels*

Material	Amount per assay tube	Nicotinic acid found	Nicotinic acid content
	cc.	$\gamma$	$\gamma$ per gm. or cc.
Urine I	0.05	0.030	0.60
	0.07	0.040	0.57
	0.10	0.055	0.55
	0.15	0.075	0.50 (0.56)
	mg.		
Rolled oats	1.0	0.010	10.0
	3.0	0.032	10.6
	5.0	0.047	9.4
	10.0	0.092	9.2 (9.8)
Liver (fresh, rat)	0.125	0.023	184
	0.175	0.031	177
	0.250	0.044	176
	0.375	0.060	160 (174)

The figures in parentheses represent averages.

*Animal Tissues*—Extraction of the finely divided tissue with a large volume of water at 15 pounds steam pressure for  $\frac{1}{2}$  hour followed by filtration has proved effective. Color or slight turbidity in the test sample does not influence the result. Preliminary autolysis of the tissue sample does not increase the extracted nicotinic acid, as is the case with biotin and pantothenic acid (18, 16). Similarly, extraction with alkali does not increase the assay value.

*Grains*—Kodicek (12) reported that grains contain substances which markedly interfere with the chemical determination of

TABLE III  
*Nicotinic Acid Content of Various Materials*

Material	Assay range	Nicotinic acid content	Recovery of added nicotinic acid
		$\gamma$ per gm. or cc.	per cent
	<i>cc. or mg.</i>		
Cattle blood.....	0.005 - 0.02	11.0	94
Yeast extract.....	0.05 - 0.2	665.0	95
Urine II.....	0.05 - 0.15	1.1	101
Corn-meal.....	10 - 50	6.0	100
Wheat flour.....	1 - 10	52.0	96
Milk.....	0.05 - 0.3	0.84	95
Liver extract.....	0.05 - 0.2	826.0	108
" (rat, fresh).....	0.125 - 0.375	174.0	100
Kidney " ".....	0.125 - 0.375	139.0	104
Heart " ".....	0.125 - 1.25	112.0	
Leg muscle (rat, fresh).....	0.125 - 1.25	76.0	
Brain (rat, fresh).....	0.125 - 1.25	58.0	
Stomach " ".....	0.125 - 1.25	68.0	
Spleen " ".....	0.125 - 1.25	64.0	
Lung (rat, fresh).....	0.125 - 1.25	42.0	
Testes (rat, fresh).....	0.25 - 1.25	30.0	
Nicotinamide.....	0.00005 - 0.001	$1 \times 10^6$	
Cozymase.....	0.0001 - 0.0002	$1.7 \times 10^6$	
Trigonelline.....	0.001 - 0.005	0.0	
Nicotinuric acid.....	0.0001 - 0.001	$7.4 \times 10^5$	

TABLE IV  
*Effect of Extraction Procedure on Nicotinic Acid Assays*

Material	Nicotinic acid content	
	Water extraction	8 per cent sodium hydroxide extraction
	$\gamma$ per gm. or cc.	$\gamma$ per gm. or cc.
Cattle plasma.....	0.90	0.82
Rat kidney.....	139.0	146.0
Rolled oats.....	14.0	15.0
Wheat flour.....	52.0	57.0
Barley flour.....	57.0	64.0
Rye flour.....	63.0	58.0
Yellow corn.....	21.0	26.0
Wheat 14279.....	56.0	67.0
" 1940.....	41.0	54.0
Corn-meal.....	6.3	10.3

nicotinic acid. Water extraction was relied upon to extract the nicotinic acid without removing these interfering substances, which were extracted by alkali treatment. We have found that when the grain is sufficiently finely ground water extraction as recommended above removes nearly as much nicotinic acid as does extraction under the same conditions with 8 per cent sodium hydroxide (grain flours, Table IV).

Through the courtesy of Professor C. A. Elvehjem we were able to obtain several samples which had been assayed for nicotinic acid by a chemical method and by assay with dogs. Comparative values obtained by the three methods are presented in Table V.

TABLE V  
*Comparative Nicotinic Acid Assays of Various Materials*

Material	Microbiological assay	Dog method	Chemical method
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Boiled ham.....	139	150	200
Liver Extract 1-20. . . . .	1320	3000	1320
Pork Liver 122. ....	534	1100	894
Peanut meal.....	172	130	
Veal Liver 70. ....	500	720	500

#### DISCUSSION

From the results presented it is evident that nicotinic acid, its amide, cozymase, or nicotinuric acid is necessary for growth and lactic acid production of *Lactobacillus arabinosus*. These compounds have equal activities when based upon their nicotinic acid content. Over a considerable range of concentrations responses to added nicotinic acid are proportional to the amount of nicotinic acid added. Landy (19), Dorfman *et al.* (13), and Pelczar and Porter (20) have shown with several bacterial species that those compounds possessing anti-blacktongue activity in dogs or anti-pellagric activity in man are effective in promoting growth in a nicotinic acid-deficient medium. This adds reliability to the use of the foregoing method for the determination of nicotinic acid or compounds of equivalent physiological activity.

The possibility that unknown factors of a stimulatory nature may cause appreciable errors in this assay seems unlikely in view

of the fact that the test organism grows luxuriantly on continued subculture in a completely synthetic medium containing only the chemically pure ingredients used in our basal medium plus nicotinic acid and amino acids.<sup>3</sup>

The possible presence of growth-inhibiting substances in natural products or extracts should not be overlooked. Recovery of nicotinic acid when added to a wide variety of materials indicates that ordinary extracts do not contain interfering amounts of inhibitory substances.

The foregoing bacteriological method is in some respects superior to existing chemical methods. No decolorization of extracts with attendant losses of nicotinic acid is required. Turbid or colored materials may be assayed without preliminary treatment. Values for grains presented in Table V are in agreement with those obtained by Kodicek for similar materials (12), and indicate that the chromogen encountered by Kodicek in his chemical method has no physiological activity. Of great value in certain cases is the sensitivity of the method, which permits its application when insufficient material is available for chemical assay.

A comparison of the several nicotinic acid assay methods as given in Table V is of interest. We attribute the higher values obtained by the dog method to the fact that these materials furnish to the ration factors other than nicotinic acid. The basal diet used in blacktongue studies is recognized to be low in factors other than nicotinic acid.

Since the test organism requires biotin for growth, the above basal medium and technique can be used also for the assay of biotin in tissue extracts simply by omitting the biotin from the medium and adding an excess of nicotinic acid (2  $\gamma$  per tube). Several assays which have been made in this manner have given results in excellent agreement with those obtained by the yeast growth method of Snell *et al.* (18); but the procedure has not been extensively investigated. Similarly, pantothenic acid is essential for this organism (14); if it is omitted from the base medium and excess nicotinic acid is added, the method becomes one for the determination of pantothenic acid. This method has not been investigated, since other acidimetric and turbidimetric assays in which lactic acid bacteria are employed are available for the

<sup>3</sup> Snell, E. E., unpublished data.

determination of this substance (16). Pyridoxine, thiamine, and riboflavin are not essential for the growth of this organism (21, 22); their addition does, however, stimulate growth during the first few hours.<sup>3</sup> The effect of purines and pyrimidines on growth of the test organism has been recently presented (23).

Generous support is hereby acknowledged from funds granted to Professor Roger J. Williams by the Rockefeller Foundation.

#### SUMMARY

An assay method for the quantitative determination of nicotinic acid in natural materials based on its essential nature for *Lactobacillus arabinosus* is presented. It is rapid, accurate, applicable to the detection of minute amounts of nicotinic acid, requires no preliminary treatment of tissue extracts, and is suitable for the routine assay of large numbers of samples.

By suitable modifications of the base medium, the procedure is also applicable to the determination of biotin and pantothenic acid.

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## THE DETERMINATION OF SERINE BY THE USE OF PERIODATE

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In our first communication (1) dealing with the reaction of hydroxyamino acids with periodic acid, we expressed the hope that procedures for the quantitative determination of such acids in protein hydrolysates might result. A very effective method for threonine (2) has been reported and we wish here to describe the results obtained with serine.

Most of the values usually given for the serine content of proteins have been obtained by actual isolation. This is a most tedious process, and very likely no one who has ever actually carried it out feels any certainty that the quantity isolated, after weeks of work, represents as much as half of that actually present.

A much more rapid, but also decidedly more speculative, method has been developed (3) in which the serine is deaminated to glyceric acid. This is either determined colorimetrically as such, or oxidized to oxalic acid. In either case, glycine interferes somewhat crucially. We have done no work with this method, and shall therefore make no further attempt to appraise it here, though it definitely deserves credit as an attempt to improve on the "isolation" method.

The method we wish to present is based on the formation of formaldehyde by the action of periodic acid on serine, and the isolation of the formaldehyde as its dimedon derivative (4) which is weighed, and may be checked for purity by the determination of its melting point.

Vorländer's procedure for the determination of formaldehyde is very satisfactory in solutions containing no other aldehydes, and no ammonia or amines. The temporary interference of ammonia,



as he found, can be overcome by longer standing, and this seems also to be the case in the presence of amino acids.

The problem of the *quantitative* isolation of the formaldehyde derivative in the presence of acetaldehyde has not previously, so far as we know, been satisfactorily solved. By control of the pH of the solution we early obtained some encouragement for the belief that a separation could be made, but the results left much to be desired.

A satisfactory solution has been obtained as follows: With essentially the method described for threonine (2) the acetaldehyde is removed as in that determination. The residual solution,

TABLE I

*Recovery of Threonine and Serine from Known Mixtures*

Each sample contained 10 mg. of threonine and 20 mg. of serine.

Threonine found	Time allowed for pptn.	Weight of dimedon ppt.	Serine found
<i>per cent</i>	<i>hrs.</i>	<i>mg.</i>	<i>per cent</i>
95.3	24	52.4	94.0
96.8	48	52.9	95.0
97.0	48	53.0	95.2
95.0	72	53.8	96.8
96.5	72	54.4	97.8
97.7	72	53.2	95.8
96.5	72	54.4	97.8
96.8	72	54.2	97.5

which now is free of acetaldehyde, is used for the precipitation of the dimedon derivative.

Table I shows the recovery of serine and threonine from a synthetic mixture. This contained, in 50 cc., 0.200 gm. of serine, 0.100 gm. of threonine, 0.100 gm. of methionine, and 1.600 gm. of alanine. Aliquots of 5 cc. were used for the determinations listed.

It should be noted that there is a consistent increase in serine found, as the time of standing is increased from 1 day to 3 days. But it is also indicated that approximate determinations may be made after 1 day.

All of these samples were aerated for 1 hour only. All of our experience shows that samples containing as much threonine as this should give values 2 to 3 per cent higher if run for another half

hour. These figures for threonine were included to illustrate the possibility of determining threonine and serine *in the same sample*. Actually, *any* sample with this much threonine should be aerated either longer or more rapidly.

These recoveries of "serine" (and all others listed in this paper) represent actual solid isolated, and do not include the correction suggested by Vorländer (4) for the solubility of the dimedon derivative. The inclusion of such a correction would raise the recovery figures by 2 to 3 per cent.

Table II presents data obtained on casein. The casein was a good commercial product which had been thoroughly extracted with ether before use. It will be noted that the serine values fall

TABLE II  
*Determination of Serine in Casein\**

Casein sample	Time of standing	Weight of ppt.	M.p. of ppt.	Serine
<i>gm.</i>	<i>hrs.</i>	<i>gm.</i>	<i>°C.</i>	<i>per cent</i>
0.625	48	0.0847	188-190	4.87
0.625	48	0.0839	188-189.5	4.82
0.625	48	0.0840	188-189.5	4.83
0.3125	72	0.0452	188-189	5.18
0.3125	72	0.0450	188-189	5.18
0.3125	72	0.0447	188-189.5	5.13

\* In certain additional experiments, known amounts of serine were added to the casein hydrolysate. In these cases, 95 to 98 per cent of the serine added was accounted for.

into two groups of three, each of which groups differs considerably in its average from the average of both groups. We have no other explanation of this difference than that of difference in time allowed for precipitation. While we are for the present willing to abide by the value of 5.0 per cent serine which the averages indicate, we believe that the ultimately accepted value will be at least 5.2 per cent. The highest value which has been reported (5) for serine by the isolation method is 0.8 per cent, with the suggestion that this value should probably be doubled.

Table III gives the results obtained for a sample of lactalbumin kindly given us by Dr. D. B. Jones, who once (6) determined serine by a revised isolation method on a different sample of this protein.

This work was probably the best that has been done on the isolation method. In contrast to their 1.76 per cent, we find 4.26 per cent.

In Table IV are shown the results of the analyses of Coignet Silver Label gelatin. The contribution made by formaldehyde derived from hydroxylysine and the question of the appraisal of this contribution are discussed later.

TABLE III  
*Determination of Serine in Lactalbumin*

Protein in sample	Weight of ppt. after standing 72 hrs.	M.p. of ppt.	Serine
<i>gm.</i>	<i>gm.</i>	<i>°C.</i>	<i>per cent</i>
0.2500	0.0298	188-189.5	4.28
0.2500	0.0288	188.5-189.5	4.15
0.2000	0.0243	188.5-189.5	4.36
0.1500	0.0178	188.5-189.5	4.27

TABLE IV  
*Determination of Serine in Gelatin\**

Gelatin sample	Weight of ppt. after standing 72 hrs.	M.p. of ppt.	"Serine"
<i>gm.</i>	<i>gm.</i>	<i>°C.</i>	<i>per cent</i>
0.4376	0.0401	188-189.5	3.29
0.4376	0.0397	188-189.5	3.26
0.3282	0.0313	188-189.5	3.43
0.3282	0.0310	188-189.5	3.39
0.5470	0.0490	188-189	3.22
0.5470	0.0489	188-189	3.22
0.3282	0.0309	188-189	3.38
0.3282	0.0300	188-189	3.29

\* We wish to make it clear that the "serine" values here recorded for gelatin do *not* represent serine only, but include (mole for mole) the hydroxylysine which is also present.

The present method for serine, which we believe to be by far the best available at the moment, has certain disadvantages, of which it will here suffice to point out two.

1. Since carbohydrates react with periodic acid to yield formaldehyde, the method can only be trusted in the absence of carbohydrates. We believe that we shall be able to avoid this difficulty, and the results, if successful, will be reported later.

2. Since hydroxylysine also yields formaldehyde with periodic

acid (7), any analysis on the total hydrolysate of a protein would probably register this acid as serine, mole for mole. As we have never had any hydroxylysine, we cannot usefully discuss further the strictly quantitative aspects of this equivalence under our reaction conditions.

But what has been said applies only to *entire* protein hydrolysates. Undoubtedly the means by which hydroxylysine has been separated will permit an easy preliminary fractionation of the hydrolysate, such that the formaldehyde determined in the separate fractions will represent essentially serine and hydroxylysine, respectively.

The results which this method for serine is able to add to the threonine method already reported furnish the basis for a very definite demonstration that hydroxyglutamic acid does not occur, to any considerable extent, if at all, in casein, in which it was discovered, and of which it is recorded as making up some 10.5 per cent. We shall analyze this situation in detail in another paper.

#### EXPERIMENTAL

*Preparation of Sample*—The protein is hydrolyzed by refluxing for 24 hours with 20 per cent HCl. The excess acid is removed as usual by repeated evaporation *in vacuo*. The portion not removable in this way does not interfere.

The hydrolysate is decolorized with a small amount of norit. This serves two purposes. The tendency of the solution to foam is so reduced that the use of nujol can be omitted during the removal of acetaldehyde. And the dimedon derivative finally obtained is decidedly purer.

#### *Procedure*

The sample, which should preferably contain 10 to 20 mg. of serine, is treated exactly as described (2) for the determination of threonine, with the single exception that no nujol is used.

It will be clear that, in a single determination of serine alone, the tubes of bisulfite could be discarded. But threonine will often be run simultaneously; and if not, it will often be desirable to run two or more determinations in series, the same gas stream being used. In such cases, the bisulfite is necessary, and each pair of determinations should have a tube of saturated  $\text{NaHCO}_3$  solution inserted between them.

After removal of the acetaldehyde the solution is transferred to a 250 cc. Erlenmeyer flask, 1 drop of methyl red indicator added, and then acetic acid drop by drop until the color changes from yellow to a faint red. The volume is now usually about 50 cc.

The formaldehyde is now precipitated as the dimedon derivative, by adding an excess of 0.4 per cent dimedon solution and allowing the stoppered flask to stand 48 to 72 hours at room temperature.

To insure complete precipitation the amount of dimedon added should be at least double that required to react with *all* the aldehydes present. In this connection, it must be recalled that *both* serine and threonine lead also to the formation of glyoxylic acid, much of which probably persists to this stage of the analysis.

After this time, the precipitate is collected on a sintered glass Gooch crucible, and dried in a vacuum desiccator. After the precipitate is weighed, its purity may be tested by determinations of melting point and of mixed melting point. We confirm the melting point of 189° for the pure derivative.

For the calculation of results, 1 mg. of dimedon derivative is equal to 0.3596 mg. of serine.

#### SUMMARY

1. A method has been described for the determination of serine in protein hydrolysates and elsewhere. Formaldehyde, formed by the action of periodate, is determined as the dimedon derivative.
2. Results on quantities of amino acids containing about 20 mg. of serine seem to be accurate to 2 to 3 per cent. This is thus much the best serine method yet described.
3. Threonine may be determined simultaneously on the same sample.

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# THE TRANSFER OF POTASSIUM ACROSS THE HUMAN BLOOD CELL MEMBRANE\*

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In 1927 Wakeman, Eisenman, and Peters (1) published data which indicated that well preserved human blood cells were impermeable to potassium and sodium salts added to blood *in vitro*, although water, chloride, and bicarbonate traversed the cell membrane. Eisenman, Hald, and Peters (2) in 1937 showed that upon the addition of sodium and potassium salts the volume of the resting cells of defibrinated blood varied directly with the concentration of base in the serum. From *in vivo* studies, however, it appeared that base crossed the cell membrane, although the volume of the blood cells acted as if it were controlled only by the osmotic pressure of the electrolytes of serum. Since the transfers of base were consistent neither in magnitude nor direction, it was suggested that base may be transferred in behalf of cellular metabolic activities rather than osmotic adjustments.

In 1940 Solomon, Hald, and Peters (3) described an increase of serum potassium of defibrinated blood maintained at 7° and 37° for 18 hours. This was accompanied by simultaneous decrease of serum sodium. The transfers occurred in both the resting and metabolically active states.

The experiments recorded in this paper deal with the movement of potassium across the human blood cell membranes at refrigerator and physiological temperatures and their relation to the carbohydrate metabolism of blood cells.

## Procedure

Blood drawn with venous stasis from subjects fasting or in the postabsorptive state was defibrinated aerobically. Cell volume

\* This article represents work done in fulfillment of the thesis requirement for the degree of Doctor of Medicine at Yale University School of Medicine.

and sugar were measured in whole blood. The serum withdrawn from blood centrifuged for approximately 20 minutes was analyzed for total proteins, non-protein nitrogen, and potassium.

A uniform suspension of cells in serum was maintained throughout all experiments by gentle agitation in the apparatus described by Heinemann and Hald (4). Rubber-stoppered 50 cc. Erlenmeyer flasks containing approximately 20 cc. of blood were oscillated ten times each minute through an arc of 60°.

To obviate the hemolysis which may follow the addition of solutes to whole blood, cells and serum were separated by a brief period of centrifuging. The supernatant serum was withdrawn and the glucose or sodium fluoride dissolved in it. Cells and serum were then remixed. Controls were subjected to an identical procedure.

Six samples of sera from blood kept at incubator temperature were examined spectroscopically for the presence of hemoglobin. Sera from deliberately hemolyzed samples of blood were used as controls.

### *Analytical Methods*

*Cell volume* was measured with Daland hematocrit tubes under the conditions recommended by Eisenman, Mackenzie, and Peters (5).

*Blood sugar* was determined by the method of Benedict (6) applied to protein-free filtrates prepared by the second zinc sulfate method of Somogyi (7).

*Serum proteins* were determined by the macro-Kjeldahl method, *non-protein nitrogen* by the micro-Kjeldahl procedure.

*Serum potassium* was determined as iodoplatinate by the method of Shohl and Bennett (8) as modified by Hald (9).

### *Results*

*Transfer of Potassium at 7°*—Blood from seven normal individuals maintained at approximately 7° was analyzed at intervals up to 48 hours (Table I). In Experiments 1 to 4 the effect of gentle agitation on the transfer of potassium was determined. Equivalent amounts of the same blood samples were placed at the same temperature without agitation to serve as controls. In the five samples studied the maximum difference in the potassium

concentration did not appreciably exceed the 2 per cent error of the method. However, despite the absence of a marked difference

TABLE I

*Transfer of Water and Potassium between Cells and Serum of Blood Kept at 7°*

Experiment No.		Hrs.	Serum proteins	Cell volume	Non-protein nitrogen	Potassium of serum	
						Observed concentration	Change per hr.
			per cent	per cent	mg. per cent	m.eq. per l.	m.eq.
1	Original		7.45	52.6	28	4.30	
	Control	18	7.59	52.7	30	7.88	
	Agitation	18	7.53	52.9	31	8.02	0.206
2	Original		8.11	47.1	35	3.54	
	Control	4	8.01	47.6	33	4.54	
	Agitation	4	7.93	47.6	34	4.42	0.220
3	Chilled 15 min.		7.29	42.2	27	3.59	
	Control	1	7.21			3.69	0.100
	"	4	7.00			3.91	0.110*
	"	18	7.12	42.7	30	5.79	0.122
	Agitation	18	7.29	42.0		5.86	0.126
4	Original		7.59	45.2		3.35	
	Control	10	7.81		31	4.83	0.148
	Agitation	10	7.69			4.90	0.155
	Control	18	7.80	45.0		6.66	0.183
	Agitation	18	7.69	44.9		6.77	0.190
5		0	7.67	42.8	29	3.75	
		4.5	7.69			4.59	0.186
		18	7.56			7.48	0.207
		24	7.63		30	8.81	0.210
		33.5	7.68			13.05	0.277
		48	7.66	43.0		17.17	0.279
6		0	7.30	48.8	39	4.01	
		48	7.65	51.1	39	15.14	0.217*
7		0	7.94	42.0	30	4.36	
		24	8.05			7.99	0.151
		48	7.91			10.13	0.120

\* Corrected for the transfer of water indicated by serum proteins.

agitation was continued throughout the succeeding experiments, since a suspension of cells would seem to be more nearly physiological than a layered sample of blood.



In the studies which were continued 18 hours, except for the slight dilution of serum protein in the 4 hour control in Experiment 3, there was no evidence of transfer of water between cells and serum. Cell volumes remained unchanged. However, in one of the experiments continued over a period of 48 hours (Experiment 6) the serum proteins increased 4.8 per cent. This concentration was probably real, since the corresponding increase in cell volume was 4.7 per cent. In no case was there a significant variation in non-protein nitrogen.

In every case potassium rose in the serum at an approximately constant rate for as long as 48 hours. The experiments were not extended beyond this time. While the transfer in a given blood sample in three instances (Experiments 3 to 5) appeared to increase at a slightly but definitely accelerated rate, the magnitude of the rise in different blood samples varied widely. However, the data are not sufficiently extensive to serve as more than an indication of the magnitude and variations which may occur. In Experiment 5 the final serum potassium values had risen to 17.2 milliequivalents which was 358 per cent above the original value.

In one experiment (Experiment 3), blood was chilled to 7° in a mixture of salt and ice during 0.25 hour; this sample was used for the determination of the initial concentration of potassium. In blood allowed in the course of 1 hour to attain equilibrium with refrigerator temperature (as determined by means of a thermometer immersed in the blood), the potassium of the serum had increased 0.1 milliequivalent. Since this point falls on the same curve as the 4 and 18 hour periods, it is clear that there is no sudden rise following the transition from room to refrigerator temperature.

#### *Effect of Incubation Followed by Storage at 7°*

Blood from two normal individuals was incubated at 37° for 4.5 hours prior to storage in the refrigerator for 18.5 and 20.5 hours (see Fig. 1).

In both experiments there was a striking drop in serum potassium during incubation for 4.5 hours. This, however, did not affect the increase of the serum potassium after the blood was transferred to the refrigerator. The curves representing the refrigerator control and the incubator-refrigerator sample parallel each other after 4.5 hours. The rate of increase in both sera was comparable,

although the curve of the incubator-refrigerator sample began at a lower level owing to the initial drop which occurred during incubation.

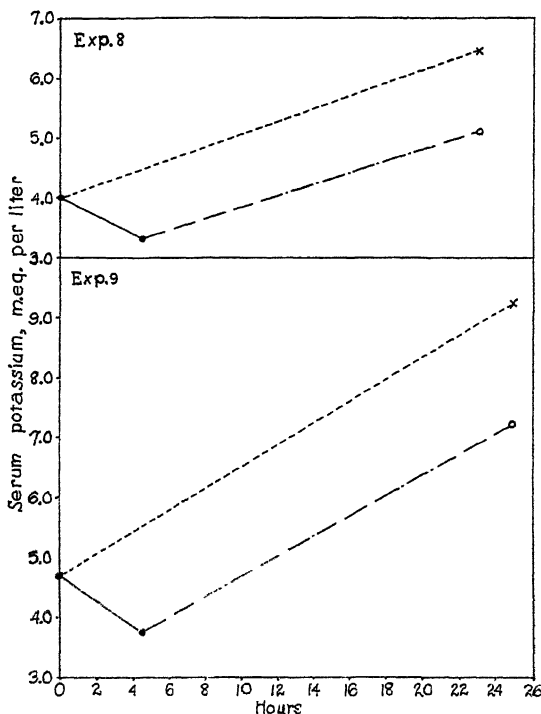


FIG. 1. The effect of incubation followed by storage at 7° on the distribution of potassium in blood (Experiments 8 and 9). The broken line indicates the course of serum potassium in blood kept at 7°. The solid line shows the change in serum potassium of blood kept at 37°. The dot-dash continuation of this line indicates the reversal of the transfer when the blood was placed in the refrigerator.

#### *Transfer of Serum Potassium at 37°*

The observation that serum potassium decreased after 4.5 hours of incubation at 37°, in contrast to the rise noted in blood kept at 7°, led to further investigation (Experiments 10 and 11) at 37°, prolonged to 18 and 13.5 hours respectively (see Table II).

As in the transition incubator-refrigerator analyses, the serum

potassium of both blood samples definitely decreased in the course of approximately 5 hours of incubation. This amounted to a disappearance from the serum of 1.16 milliequivalents of potassium per liter in the first instance and 0.64 milliequivalent per liter in the second. A determination at the 2.5 hour interval in Experiment 11 suggested that this was a gradual rather than a sudden process.

TABLE II

*Transfer of Water and Potassium between Cells and Serum of Blood Kept at 37°*

Experiment No.	Hrs.	Serum proteins	Potassium of serum	
			Observed concentration	Absolute change
		<i>per cent</i>	<i>m.eq. per l.</i>	<i>m.eq. per l.</i>
10	0	7.85	4.17	
	4.5	7.93	3.01	-1.16
	7	8.12	3.21	-1.07*
	18	8.59	10.80	5.71*
11	0	7.59	5.54	
	2.5	7.18	4.77	-0.50*
	5	7.23	4.67	-0.64*
	13.5	8.05	9.47	3.39*

\* Corrected for the transfer of water, indicated by the change in the concentration of serum proteins.

The 13.5 hour and 18 hour serum potassium values established that not only had potassium returned to the serum but that the original values had been surpassed by 53 and 61 per cent.

This latter phase was accompanied in both instances by a concentration of serum proteins. In Experiment 11, however, the serum proteins became diluted during the initial decrease of potassium.

It would seem, therefore, that during the first 5 hours of incubation at 37° potassium leaves the serum and enters the cells. This process is reversible, since the potassium returns to the serum at a rapid rate when incubation is prolonged beyond 5 hours.

*Glycolysis in Relation to Transfer of Potassium*

The consistent decrease in serum potassium of blood incubated 4 or 5 hours (see Fig. 1 and Table II) suggested an investigation of this change in relation to glycolysis.

After 2.5, 8.5, and 10 hours of incubation, glucose was added to samples of the same blood in quantities large enough to raise the concentration of sugar by 160 mg. per cent. The control blood was subjected to the same remixing process without the addition of glucose.

Adding glucose to blood after 2.5 hours of incubation definitely suppressed the rise of serum potassium which occurred in the control. In Experiment 12 (see Table III) it is evident that the curves representing the level of serum potassium in the control and the blood to which glucose had been added began to diverge at 8.5 hours. They are widely separated at 16.5 hours. The potassium in the control serum had risen from 4.1 to 8.0 milliequivalents, while that in the sample containing added glucose failed to regain its original concentration. A similar, although quantitatively less marked response was elicited in Experiments 13 (see Table III) and 14 (see Fig. 2) in which glucose was added after 10 and 8.5 hours of incubation, respectively. In both instances, at the time glucose was added the potassium in the serum was increasing. At the same time swelling of the cells was indicated by increased cell volume and serum protein.

Blood glucose was followed in Experiments 15 (see Table III) and 16 (see Fig. 3). From these determinations it appears that glycolysis was complete after 5 hours in blood withdrawn from normal subjects in the postabsorptive state with initial levels of 84 and 82 mg. per cent. In both studies the completion of glycolysis coincides with the lowest serum potassium concentration. In Experiment 16 in which glucose had been added at 2.5 hours, the initial decrease in serum potassium was maintained until the end of the study. Blood sugar values of 134 mg. per cent at 9 hours and 80 mg. per cent at 14.75 hours indicate that glycolysis was still proceeding. At this time in the control blood the rise in serum potassium had progressed to 7.0 in contrast to the 3.7 milliequivalents observed in the experimental blood. In the latter sample, the increase in cell volume and serum protein

concentration at the end of incubation was 40 per cent less than that observed in the control.

In an attempt to inhibit glycolysis sodium fluoride was added to samples of blood. Concentrations of sodium fluoride of 0.05

TABLE III

*Transfer of Potassium between Cells and Serum in Relation to Glycolysis, As Measured by Its Concentration in Serum after Varying Intervals at 37°*

Ex- peri- ment No.		Control blood						Addition to blood	
								Glucose at 2.5 hrs.	
12	Hrs.	0	2.5	5	8.5	16.5		8.5	16.5
	Cell volume, %	40.7				51.8			
	Serum proteins, %	8.23	7.9	8.21	8.19	9.07		8.16	8.82
	" K, m.eq. per l.	4.1	3.4	3.1	4.0	8.0*		2.9	3.3*
								Glucose at 10 hrs.	
13	Hrs.	0	4	10	19			19	
	Cell volume, %	43.8			52.0				
	Serum proteins, %	7.49	7.50	8.05	8.57			8.55	
	" K, m.eq. per l.	4.5	3.6	6.3*	10.5*			7.3*	
15	Hrs.	0	2.5	4.5	5.5	7	15		
	Serum proteins, %	6.78			6.82	7.05	7.35		
	" K, m.eq. per l.	4.5			3.5*	3.6*	5.6*		
	Blood sugar, mg. %	84	49	10†		0†			
								0.05 per cent NaF at 0 hr.	
17	Hrs.	0	2.5					2.5	
	Serum proteins, %	6.35	6.37					6.41	
	" K, m.eq. per l.	4.0	3.5					6.1	

\* Corrected for the transfer of water by serum proteins.

† Value derived by subtracting the reading of the blank determination.

and 0.025 per cent were found to retard glycolysis effectively without causing hemolysis. It was found by two experiments that when a concentration of 0.05 per cent was added no change in blood sugar was apparent after 7 hours at 37°. Spectroscopic analysis at 2.5 hours showed no hemolysis. When 0.025 per cent sodium fluoride was used, there was a drop in blood sugar at the rate of 4 mg. per hour in one case; in the other no change was noted. After 8 hours at 37° no indication of hemolysis could be detected.

In contrast to controls in which the serum potassium regularly decreased initially, the potassium in the two samples containing

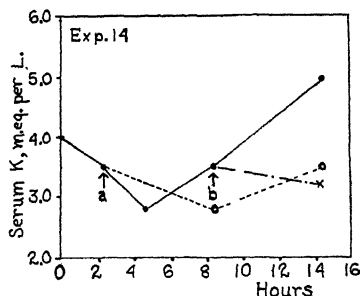


FIG. 2. The effect of adding glucose upon the distribution of potassium in blood kept at 37° (Experiment 14). The solid line represents the course of potassium in the serum of blood kept at 37°. The broken line shows the effect of adding glucose at the time indicated by the arrow *a*; the dot-dash line, the effect of adding glucose at the time indicated by the arrow *b*.

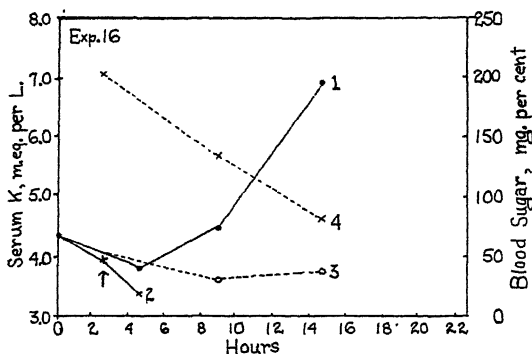


FIG. 3. The effect of adding glucose upon the distribution of potassium in blood kept at 37° (Experiment 16). The solid lines indicate the course of serum potassium (Curve 1) and blood sugar (Curve 2) in the control blood; the broken lines, the course of serum potassium (Curve 3) and blood sugar (Curve 4) in the blood to which glucose was added at the time indicated by the arrow.

sodium fluoride rose sharply from the beginning, Experiment 17 (see Table III) and Experiment 18 (see Fig. 4).

The blood for Experiment 18 was taken from a mildly diabetic patient with a postabsorptive blood sugar of 157 mg. per cent. This patient was not receiving insulin. A comparison of the

control potassium curve of this blood with that of the non-diabetic bloods used in all previous incubator experiments reveals that, although similar in its contour, the initial decline extends over a longer period of time; the original level is not regained until after 12 hours of incubation. The results of the previous experiments suggest that in this blood glycolysis was not complete until 10 to 12 hours of incubation. This is not an unlikely assumption, since it implies that glycolysis progresses at approximately 15 mg. per cent per hour, a rate which falls close to the average observed in

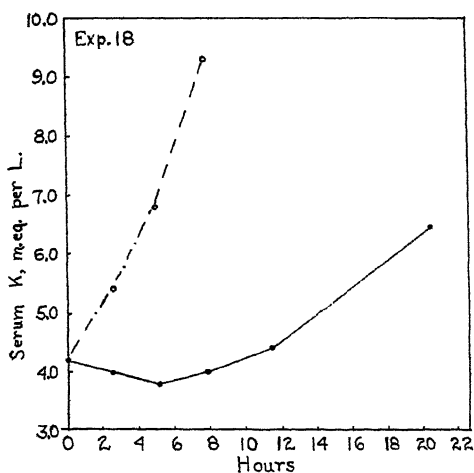


FIG. 4. The effect of sodium fluoride on the distribution of potassium in blood kept at 37° (Experiment 18). The solid line represents the course of serum potassium in the control blood; the dot-dash line, the course of serum potassium in blood to which fluoride had been added.

both diabetic and non-diabetic blood by MacLean (10). Had this experiment been terminated at 12 or 14 hours, the curve would resemble those of Experiments 12 and 14 in which glucose was added at 2.5 hours to normal blood.

#### DISCUSSION

The transfer of potassium between serum and cells appears to be different at physiological and refrigerator temperatures. At 7° blood cells gave up potassium to the serum at an almost constant rate for as long as 48 hours. At the end of this period the concentration of potassium in the serum had increased 3- or 4-fold. The

cell volume, however, did not change. From the earlier experiments of Solomon, Hald, and Peters (3) it would appear that, during the first 18 hours, at least, this transfer of potassium is balanced by a reciprocal transfer of sodium.

When blood was kept in the incubator, not in the refrigerator, serum potassium, instead of increasing, diminished sharply, evidently entering the cells. When the blood was transferred to the refrigerator again at the end of 4.5 hours, however, the flow of potassium was reversed; serum potassium increased steadily. The rate of increase was the same as that observed in blood that was kept in the refrigerator from the beginning, although it started from a lower initial point. The latter part of the curve proves that incubation has not altered the inherent characteristics of the blood. In the incubator metabolic activities were providing energy. The potassium which enters the cells may be required for the chemical reactions involved in these metabolic activities. Potassium could be secured, however, from the serum only by increasing the concentration gradient across the membrane. It may be something more than coincidence, then, that potassium entered the cells during metabolic activity. When this was checked by cold, the current reversed and potassium gradually leaked out of the cells again along the resting curve. During neither of these processes did the volume of the cells change appreciably. In the cold it has been pointed out that potassium and sodium exchanges balanced one another. Whether this is true of the incubator experiments remains to be ascertained.

When blood was kept in the incubator for a longer time it was found that the movement of potassium into the cells was self-terminative. At the end of 5 or 6 hours it ceased and shortly thereafter potassium began to pour out of the cells again, at a rate greatly in excess of that at which it escaped in the refrigerator experiments described earlier. Further experiments demonstrated that the reversal of the potassium current coincided with the completion of glycolysis. It marked the point at which glucose in the cells was exhausted and the phosphate esters began to break down. This is clear from the demonstration that the discharge of potassium could be delayed by the addition of glucose. Furthermore, when glycolysis was inhibited by means of fluoride, the discharge of potassium from the cells occurred from the very outset of the experiment.



In the incubator, when potassium began to leave the cells, after glycolysis was complete, the cells swelled progressively, taking water from the serum. This distinguishes this particular reaction from all the others. The difference in this respect from the slow degradation process at refrigerator temperature cannot be attributed to the effects of temperature. It seems to be connected with the chemical reactions within the cells, quite probably with the demolition of the phosphate esters and glucose. In the fluoride experiments, in which these processes were checked quite as effectively as they were by chilling, cell volume did not change, although the blood was kept at 37°.

Since this study was completed, Eisenman, Ott, Smith, and Winkler (11) have shown that, if radioactive potassium is added to blood, no significant radioactivity can be detected within the cells for as much as 4 hours at 37°. The failure to detect any transfer of potassium seems to contradict the studies under consideration. However, the transfers here reported under the experimental conditions observed by Eisenman, Ott, Smith, and Winkler are extremely small, and negligible in comparison with the large amounts of potassium salts that were added to the blood. Movement of a small quantity of total potassium would, therefore, have an inappreciable effect upon the radioactive potassium, since the latter makes up only a small fraction of the whole. It can, however, be inferred that the potassium in cells and in serum is not in diffusion equilibrium, even when potassium is passing in or out of the cell, while the latter is chemically intact. This suggests that definite quantities of the potassium on one side of the membrane or the other are moved across the boundary in connection with specific reactions that are, under any given circumstances, unidirectional. Under any other conditions greater admixture of potassium between the two media would be inevitable.

It is possible that in some of these transfers only a part of the blood cells or a particular type of blood cell is involved.

#### SUMMARY

1. The effects of temperature, glycolysis, and sodium fluoride upon the distribution of potassium between cells and serum *in vitro* have been investigated.

2. At 7° potassium escaped from the cells into the serum at an

approximately constant rate for as long as 48 hours. No transfer of water was apparent. At 37° potassium entered the cells during the first 5 hours. It then reversed its direction and poured out into the serum. During the first period no change of water was noted but as the potassium subsequently entered the serum, cell volume increased.

3. The reversal of the current of potassium coincided with the point at which glycolysis was complete. Prolonging the period of glycolysis by the addition of glucose delayed this reversal.

4. The addition of sodium fluoride at 37° resulted in the rapid transfer of potassium from cells to serum continuously from the outset. There was no change in cell volume.

5. The implications of these findings have been discussed.

The writer is indebted to Miss Pauline M. Hald of the Department of Internal Medicine for her counsel and guidance in this work.

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## ON THE COLORIMETRIC DETERMINATION OF VITAMIN B<sub>6</sub>\*

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Color reactions of vitamin B<sub>6</sub> fall into two groups; namely, those which depend upon the phenolic nature of the vitamin, and those which depend upon other portions of the molecule. The reactions with the Folin-Denis reagent, ferric chloride, and diazotized sulfanilic acid fall into the first group. Kuhn and Low (4) reported that the coupling reaction was unsuited to the determination of the vitamin. In a preliminary communication, Swaminathan (5) reported on the use of this reaction for the estimation of vitamin B<sub>6</sub> in foodstuffs. The lack of specificity of the reactions in this first group, however, leaves much to be desired.

Included in the second class of color reactions are the formation of cyanine dyestuffs and of phthaleins. The preparation of the cyanines depends upon the reactivity of the  $\alpha$ -methyl group of the vitamin and requires a preliminary conversion of the vitamin to a phenolic ether. This preparation is difficult and the yields are not quantitative. Stiller, Keresztesy, and Stevens (6) oxidized the vitamin to the 4,5-dicarboxylic acid and fused the resultant acid with resorcinol to obtain the corresponding fluorescent phthalein. Although important in their elucidation of the structure of vitamin B<sub>6</sub>, this reaction is not suited to the determination of the vitamin, since the reaction is neither quantitative nor specific.

Stiller, Keresztesy, and Stevens (6) reported that the vitamin reacts with 2,6-dichloroquinonechloroimide. The blue reaction product is obtained only at high vitamin concentrations, and

\* This method was described briefly in a preliminary communication (1) and has been used to study the urinary elimination of vitamin B<sub>6</sub> in the mouse (1) and the dog and human subject (2, 3).

the colors are very transient. We have studied this reaction and have devised a colorimetric method for the determination of the vitamin which appears to possess a good sensitivity and specificity together with a satisfactory degree of stability. Details of this study are presented here. The application of the method to biological material is still under investigation, but data of general interest in this connection are presented herein.

*Reagents—*

1. Chloroimide reagent. 100 mg. of 2,6-dichloroquinone-chloroimide (Eastman, No. 2483) were dissolved in 1600 cc. of acid-free reagent grade normal butanol. The reagent was stored in a refrigerator in a brown bottle. Samples withdrawn daily were allowed to warm to room temperature before use. Under these conditions the reagent was stable for at least 2 weeks. To determine whether a reagent was satisfactory, pure butanol was used to indicate 100 per cent transmission in the Evelyn colorimeter with filter No. 660. Control tests run in the absence of vitamin gave an absorption of 14 to 16 per cent. Reagents which gave an absorption of 20 per cent or more were discarded.

The solid chloroimide was crystallized from dry benzene by the addition of petroleum ether (m.p. 65.5–66° corrected). Comparison of this material directly with several Eastman samples indicated that ordinarily this is not a necessary precaution.

2. Half strength chloroimide reagent. Equal volumes of the standard chloroimide reagent and butanol were mixed.

3. Veronal buffer, pH 7.6. This was prepared by dissolving 15 gm. of sodium diethylbarbiturate (Merck) in 700 cc. of distilled water and titrating to pH 7.6 with the glass electrode. The solution was filtered from the precipitated diethylbarbituric acid.

4. Borate buffer. This was the usual Clark and Lubs buffer at pH 8.6.

5. Sodium hydroxide and hydrochloric acid solutions. These were made up in concentrations of approximately 0.1, 1.0, and 30 per cent.

6. Brom-thymol blue and thymol blue solutions. These were made up by dissolving 100 mg. of the dye in a minimal volume of alcohol and diluting to 1000 cc. with water. The solutions were then adjusted to their mid-points with dilute acid or alkali.

*Method*

Aqueous solutions of the vitamin were adjusted to a pH of 6.8 to 7.2, with brom-thymol blue as an external indicator.

To a neutral aqueous solution of the vitamin 1 volume of the veronal buffer and 4 volumes of the butanol solution of the reagent were added. The reaction mixture was briefly but vigorously shaken. This was repeated after 5 minutes, and at the end of 15 minutes the two phases were separated by centrifuging. 3 volumes of the supernatant butanol, which contains the vitamin indophenol, were pipetted into 1 volume of absolute alcohol. The colors were read 50 ( $\pm 5$ ) minutes after the addition of the reagent.

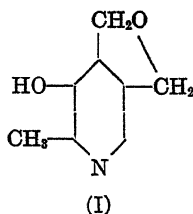
At laboratory temperatures of 20–24° the intensity of the color reached a maximum within 40 minutes and remained constant between 40 and 60 minutes after the addition of the reagent. However, the reaction possesses a large temperature coefficient. It is, therefore, advisable to check the rate of color development and the time interval over which the color is stable.

The range of vitamin concentrations to which the test is applicable varies with the instrument used. With a visual comparator, concentrations of 10 to 40  $\gamma$  of the vitamin per cc. of test solution give colors sufficiently intense for comparison. When read in the Bausch and Lomb spectrophotometer at 650  $m\mu$ ,<sup>1</sup> solutions containing 2 to 40  $\gamma$  of the vitamin give colors which obey Beer's law. For solutions containing 0.5 to 10.0  $\gamma$  of the vitamin the Evelyn colorimeter with filter No. 660 was used. In such dilute solutions, it was found desirable to use the half strength chloroimide reagent, and an increased sensitivity was obtained by drying the butanol phase with anhydrous sodium sulfate and reading directly without further dilution. A great many determinations made with both of these instruments have shown that comparisons can be made within the limit of the reading error; that is, within 2 to 5 per cent.

<sup>1</sup> In aqueous solution indophenols usually show maximum absorption at 610  $m\mu$  (7), but both the vitamin indophenol and 2,6-dichloroindophenol show a shift to 650  $m\mu$  when the solvent is butanol or a 3:1 butanol-ethyl alcohol solution. The curves, however, are similar in disposition.

Vitamin B<sub>6</sub> and boric acid have been shown to undergo complex formation (8) in which 2 molecules of the vitamin are linked to boron through the 3-hydroxyl and 4-hydroxymethyl groups. These linkages mask the phenolic nature of the vitamin and inhibit the indophenol reaction. Consequently, the use of a borate buffer permits a differentiation of vitamin B<sub>6</sub> and extraneous phenols. A solution which gives the indophenol reaction in the presence of a borate buffer contains interfering substances. Means of eliminating such substances are discussed later.

Samples were routinely analyzed in duplicate, a veronal buffer being used in one tube and a borate buffer (pH 8.6) in the other. The latter test was run as indicated, with the exception that the one-half strength reagent was always used. For this test, solutions were always diluted to contain less than 50  $\gamma$  of the vitamin per cc. This is necessary because excessive concentrations of the vitamin give a small amount of indophenol formation with this borate buffer as a result of a slight dissociation of the borate complex. By performing duplicate tests in this way it is possible to detect extraneous phenols (0.5  $\gamma$  per cc.) in the presence of the vitamin (50  $\gamma$  per cc.).



The rate of reaction of the reagent and a given phenol at a given pH differs with different buffers. With the inner ether of vitamin B<sub>6</sub> (I) it was found that a veronal buffer at pH 7.6 gives the same intensity and rate of color development as a borate buffer at pH 8.6. When phenol was similarly studied, it was found that these buffers were approximately equivalent under the test conditions. Because different phenols react at different rates with these buffers, and because certain non-vitamin phenols may react in the presence of a borate buffer, it has not been possible to determine the concentration of vitamin B<sub>6</sub> in mixtures of phenols by simply subtracting the concentration found when borate buffer is used from

that obtained when veronal buffer is used. The use of the borate buffer is restricted to the recognition of interfering substances in analytical samples. When present, these must be eliminated.

*Reaction Mechanism*—In studies of the indophenol reaction Gibbs (7) observed that quinonechloroimides were decomposed by alkalis. The rate of decomposition was increased with increasing pH and was accelerated by light. Hydrolysis to the quinone oxime was shown to be the major reaction, although not the only one. Using an excess of hydroxyl ion in the form of a borate buffer, Gibbs obtained a second order velocity constant at

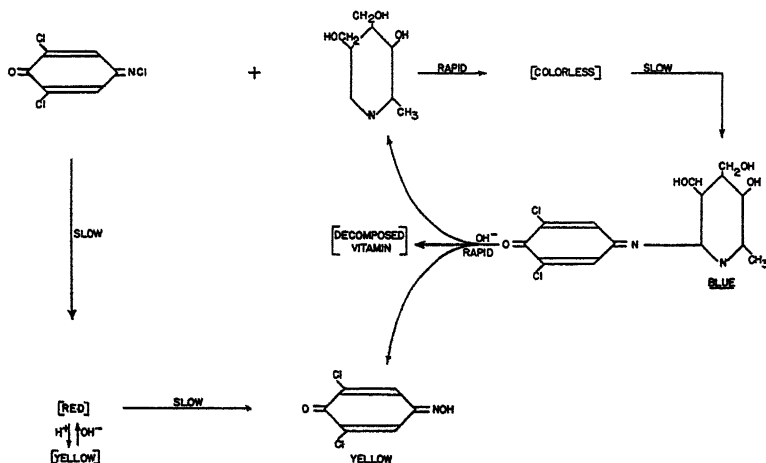


FIG. 1. Reactions occurring in the butanol phase

equal concentrations of the reagent and phenol, and on increasing the chloroimide concentration a first order constant. On a kinetic basis Gibbs postulated an intermediate, colorless, phenolic ether rearranging to the blue indophenol.

A similar study has not been attempted here because of complications introduced by the 2-phase system, and because of an intermediate rate-controlling reaction which occurs in the course of the development of the vitamin indophenol. The reactions shown in Fig. 1 were demonstrated by experiments carried out under conditions similar to those used in the test procedure.



*Decomposition of 2,6-Dichloroquinone*—When the test is performed in the absence of the vitamin, the reagent slowly generates an unknown red tautomeric substance in the butanol phase. This substance shows a broad absorption band at 540 m $\mu$ . The optical density of the color is proportional to the concentration of the reagent and increases slowly with time. The density of this color at a given time is proportional, within limits, to the pH of the buffer used; for example, increasing the pH of a veronal buffer from 7.6 to 9.8 increases the color. This increase in color is, however, a function of the anion as well as the pH, since at a pH of 8.5 the density of the red color produced in the butanol phase is greatest with veronal, less with phosphate, and least with a borate buffer.

Given sufficient time, or upon the addition of alkali to the aqueous phase, this red color disappears, and the reagent is converted to the yellow-colored quinone oxime, part of which migrates into the water phase.

By the use of a low pH, and a minimum concentration of the chloroimide, these side reactions are reduced to negligible proportions. Their influence is further reduced by the use of filters. It is advisable, however, to discard old or decomposed solutions of the reagent, since these may give rise to excessive amounts of these by-products.

*Formation of Colorless Intermediate*—When a solution of vitamin B<sub>6</sub> in a veronal buffer was shaken at pH 7.6 with butanol for 1 minute and the two phases separated, very little of the vitamin was extracted. When the experiment was repeated with a butanol solution of the reagent, the vitamin was almost completely removed. Since the solvent characteristics of the butanol can hardly be changed by the presence of 50 mg. of the reagent per 800 cc. of butanol, this is experimental evidence of a rapid reaction. The colorless reaction product rearranged more slowly to the blue indophenol in the butanol phase.

*Formation of Vitamin Indophenol*—Buffer anions are significant in the synthesis of the vitamin indophenol. A veronal buffer at pH 8.5 produces a rate of color development essentially equal to that of a phosphate buffer at pH 9.2, whereas the reaction does not proceed at all in the presence of a borate buffer. For a

given buffer<sup>2</sup> the rate increases with pH. An optimum is reached beyond which the rate of decomposition of the vitamin indophenol is accelerated.

*Decomposition of Vitamin Indophenol*—Although the vitamin indophenol is markedly stabilized by its preparation in butanol,<sup>3</sup> it has a limited stability. Its rate of decomposition varies directly with the pH and time. With a veronal buffer at pH 7.6, decomposition of the vitamin indophenol is not complete within 8 hours, whereas the decomposition can be completed within 10 to 15 seconds by the addition of strong alkali.

The decomposition of the vitamin indophenol is a composite of at least three reactions. The essential excess of the reagent gives rise to the unknown red product, and this material then gives rise to the quinone oxime. At the same time the vitamin indophenol is decomposed. These reactions can be followed spectrophotometrically, since the decomposition of the reagent is slower than the decomposition of the vitamin indophenol. Fig. 2 shows the spectrophotometric data obtained. In the first experiment (Curve 1) a blank test was performed and, 40 minutes after the addition of the reagent, alkali was added to a pH of 9 to 10 in the water phase. The reaction mixture was separated and the color generated in the butanol phase was examined in the Bausch and Lomb spectrophotometer after 45 minutes. The red color is that produced intermediate to hydrolysis of the reagent to the quinone oxime. In the second experiment (Curve 2), the test was performed with a solution containing 20  $\gamma$  of vitamin B<sub>6</sub> per cc. After 40 minutes, the reaction mixture was treated with alkali as in the first experiment, thus destroying the blue color of the vitamin indophenol. Curves 1 and 2 are identically placed, but the reaction involving the vitamin gave a smaller amount of the red product. This decrement can be quantitatively accounted for on the basis of the reactions given in Fig. 1. One-twelfth of the reagent is bound as the vitamin indophenol. By hydrolysis

<sup>2</sup> Unlike indophenol reactions carried out in aqueous solution, the vitamin indophenol is formed in the butanol phase even if the water phase is slightly acidic (pH 6.0).

<sup>3</sup> The vitamin indophenol is formed in the absence of water when the vitamin, the chloroimide, and sodium veronal are mixed in dry butanol.

of the vitamin indophenol, one-twelfth of the reagent is converted directly to the quinone oxime and is therefore incapable of producing the red color.

In a similar pair of experiments strong alkali was added to the reaction mixtures. In the blank test (Curve 3) the curve obtained

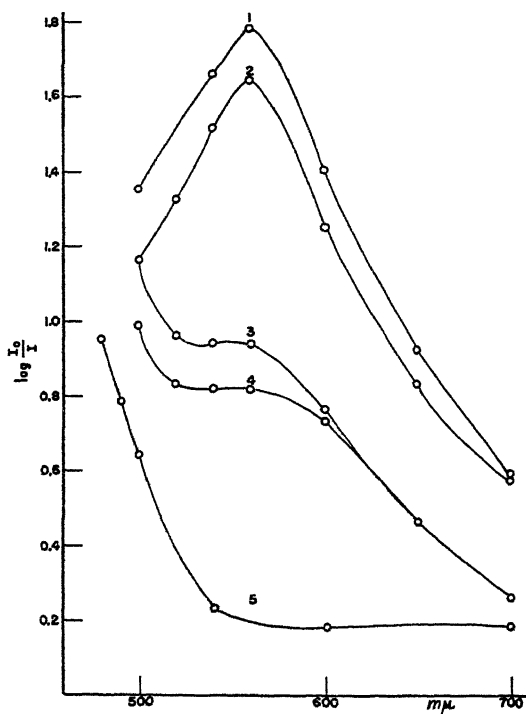


FIG. 2. Curve 1, test run in absence of vitamin B<sub>6</sub>, alkali added to pH 9 to 10, 40 minutes after the reagent was added; Curve 2, same as for Curve 1 in the presence of 20  $\gamma$  of vitamin B<sub>6</sub> per cc.; Curve 3, same as for Curve 1, strong alkali added; Curve 4, same as for Curve 2, strong alkali added; Curve 5, butanol solution of 2,6-dichloroquinone oxime. These data were obtained with a 10 cm. tube, whereas the test is run in a 1 cm. cuvette.

was flat between 520 and 560 m $\mu$ . This results from admixture of the quinone oxime and the red-colored intermediate. Curve 4 (the test solution containing 20  $\gamma$  of the vitamin per cc.) is similar to Curve 3, but again the optical density of the solution is depressed by one-twelfth.

According to these data, the vitamin may be regenerated, or converted to essentially colorless products. Large scale experiments designed to isolate the regenerated vitamin were performed. These failed to show more than 50 per cent of the vitamin regenerated. Consequently, it appears that the vitamin is altered in part.

This lability of the vitamin indophenol to alkali is somewhat characteristic, since the corresponding phenol indophenol and indamine are stable to alkali. An indophenol which is not decomposed by alkali is not the vitamin indophenol.

*Interfering Substances*—It is generally assumed that chloroimide reagents are specific to phenols which are unsubstituted in the para position. However, Raybin (9) reported that vitamin B<sub>1</sub> gave a yellow color with 2,6-dichloroquinonechloroimide, and attributed this color to the thiazole part of the molecule. A number of apparently unrelated substances give colors with the chloroimide reagent under the present test conditions; for example, creatine, creatinine, phenylhydrazine, and phenylhydroxylamine give yellow colors, whereas uric acid and carbon disulfide form yellow to pink colors, and furfuraldehyde produces a green color. As is well known, certain aromatic amines give rise to indamine formation, and phenols other than vitamin B<sub>6</sub> may interfere.

The indophenols are oxidation-reduction indicators. Reducing agents such as hydrogen sulfide, cysteine, hydroquinones, ascorbic acid, etc., must therefore be eliminated. Oxidizing agents such as alloxan must also be eliminated.

*Elimination of Interfering Substances*—Phenols other than vitamin B<sub>6</sub> may react in the present test, but the effect of these has been reduced by the use of a 2-phase system, and a low pH; for example, free phenol will not interfere with the measurement of 5  $\gamma$  of vitamin B<sub>6</sub> per cc. unless the concentration of phenol exceeds 50  $\gamma$  per cc. Some phenols, such as resorcinol, give indophenols which can be readily removed from the butanol phase by extraction with additional buffer. These phenols may or may not interfere, depending on their concentrations, but in either case their presence is readily detected. Some water-soluble phenols, as for example the 4,5-dicarboxylic analogue of the vitamin, give indophenols which do not migrate into the butanol phase. Nitrogen substitution in the vitamin molecule, as in the N-methyl derivative, completely inhibits the reaction. Thus,

the above two substances, both closely related to the vitamin structurally, do not interfere in the determination.

Many interfering substances can be eliminated by simple procedures; for example, volatile and ether-soluble phenols, aldehydes, acids, lactones, etc., can be extracted from acidic solutions of the vitamin with immiscible solvents prior to performing the test. In a control series of experiments, solutions of the vitamin at pH 1 to 2 were extracted three times with equal volumes of water-saturated ether or butanol with no measurable loss of the vitamin.

The solubility and other properties of water-soluble, non-volatile phenols are altered to advantage by treatment with nitrous acid. In a control series of experiments, solutions containing varying vitamin concentrations in mineral acid were treated at 100° for 10 minutes with 10 per cent sodium nitrite. The vitamin was recovered in 100 per cent yields. This treatment serves to decompose amines, which can then be extracted, along with many water-soluble phenols and other colored substances, from the acidic solution with water-saturated butanol.

Purines, other nitrogenous bodies, and reducing substances can be removed by treatment with silver salts. The vitamin is not precipitated from acidic solution (pH 3 to 5) by silver lactate, and after removal of the silver as the chloride, 100 per cent recoveries of added vitamin were obtained. The use of baryta to remove excess silver gave variable recoveries of 65 to 85 per cent of the added vitamin.

*Stability of Dilute Solutions of Vitamin*—When autoclaved in neutral solution, appreciable quantities of the vitamin undergo autocondensation (8). Harris<sup>4</sup> has isolated an autocondensation product in which 2 molecules of the vitamin are linked together. This product gives an indophenol reaction equivalent only to 1 molecule of the vitamin. This condensation product simulates the vitamin in that it gives a negative indophenol reaction in the presence of a borate buffer. This reaction would be negative if the 3-hydroxyl and 4-hydroxymethyl groups in the nitrogen-substituted part of the molecule were free.

It has been possible to measure, by the present test procedure, the amount of autocondensation which occurs in hot neutral

<sup>4</sup> Harris, S. A., unpublished data.

solution. Samples of the condensation products were isolated and tested as usual. By comparison with equivalent weights of the vitamin it was observed that these usually involved mixtures of di- and trimolecular products. After neutral solutions of vitamin B<sub>6</sub> were autoclaved, the solution was tested colorimetrically. The color measured is a result of two indophenol reactions; namely, that involving unchanged vitamin B<sub>6</sub>, and its condensation products. The over-all concentration is always less than 100 per cent of the vitamin originally present and this decrement results from the autocondensation. Assuming that the condensation products remaining in solution have the same molecular weights as those isolated, this decrement divided by a factor for the molecular weight of the condensation product gives the percentage of the vitamin converted to the condensation product. In this way, it was found that autocondensation was more extensive in 10 than in 1 per cent solutions of the vitamin and, in agreement with the zwitter ion nature of the vitamin (10), the condensation was more pronounced at pH 7 than at pH 6. As much as 35 per cent of the vitamin can be destroyed (these condensation products are without vitamin activity (8)) on autoclaving a 10 per cent solution of the vitamin at 20 pounds pressure for 20 minutes at pH 7.

These findings suggest that treatment with water (pH 7) at elevated temperatures might not be satisfactory for the extraction of the vitamin from natural sources.

Vitamin B<sub>6</sub> is not stable to strong acids at elevated temperatures. Solutions of the vitamin hydrochloride (100 mg. per cent) may be autoclaved at 20 pounds pressure with no decomposition for periods of at least 2 hours. In the presence of 6 N HCl, however, about 1 per cent of the vitamin is dehydrated and converted to the inner ether (I) within 30 minutes, and within 2 hours over 20 per cent of the vitamin is converted to this biologically inactive material. The conversion of the vitamin to this product was measured directly by the indophenol reaction given in the presence of a borate buffer. Salt concentrations must be kept below 0.5 per cent to prevent deviations from Beer's law when solutions containing less than 5  $\gamma$  of (I) are measured. In the presence of a veronal buffer, solutions of the vitamin which were previously heated with strong acid appear to give greater than 100 per cent

recoveries of added vitamin. This anomalous recovery results from the formation of the inner ether (I), since this ether gives a greater intensity of color in the indophenol reaction than an equivalent weight of vitamin B<sub>6</sub>. This greater intensity may be due in part to a more complete conversion to the indophenol, but among other things, it is certainly due to an increased stability of the indophenol once it is formed.

In connection with the acid hydrolysis of protein material, a similar destruction of the vitamin was observed upon refluxing the vitamin in constant boiling hydrochloric acid.

Another type of reactivity of vitamin B<sub>6</sub> was studied which is of interest in connection with the use of a continuous butanol extraction of the vitamin from dilute solutions of biological fluids. Refluxing dilute solution of the vitamin base in butanol (bath temperature, 200°) for 16 hours converted as much as 50 to 65 per cent of the vitamin to a substance which gave a positive indophenol reaction in the presence of a borate buffer. At a bath temperature of 100° only 5 per cent of the vitamin was converted to this substance in an equal period of time. The product is probably the 4-butyl ether of the vitamin. This compound has now been synthesized by Harris.<sup>4</sup>

*Concentration of Samples for Analysis*—The vitamin is removed unchanged from dilute neutral solutions by continuous butanol extraction if the extraction is carried out under reduced pressures at bath temperatures of 80–90° or lower. Under these conditions, it has been possible to recover 100 per cent of the vitamin from dilute aqueous solutions (5 and 10  $\gamma$  per cc.) within 12 to 14 hours when the butanol was refluxing at a rate of 3 drops per second.

The vitamin is completely removed from neutral solution by zeolite, and consistent amounts of the vitamin have been eluted as follows: 10 gm. of 30 mesh zeolite in 10 mm. glass tubing were washed with 50 cc. of 5 per cent acetic acid and 100 cc. of water. Solutions of the vitamin (100 cc.) were put through the column at the rate of 1 drop per second. The column was washed with 50 cc. of water and the vitamin was eluted with 50 cc. of boiling 10 per cent KCl. In a series of sixteen experiments with solutions containing 10 to 40  $\gamma$  per cc., an average recovery of 91 per cent ( $\pm 2$ , maximum deviation 6 per cent) of the vitamin was obtained.

With vitamin concentrations of 1 to 3  $\gamma$  per cc. the salt concentration in the eluate caused slight deviations from Beer's law.

#### DISCUSSION

The method for the determination of vitamin B<sub>6</sub> with 2,6-dichloroquinonechloroimide has many marks of specificity. These are as follows: The reaction is given only by phenols unsubstituted in the para position. At the pH used, high concentrations of free phenol do not react within the time limit imposed. By means of the 2-phase system, many water-soluble phenolic substances, including the 4,5-dicarboxylic analogue of the vitamin, do not interfere because of their solubility in the alkaline aqueous phase. Certain phenols, such as resorcinol, may interfere, but if their concentrations are not too large the interfering colors can be washed out of the butanol phase. The use of immiscible solvents permits the removal of interfering substances from acidic solutions of the vitamin, as well as the quantitative extraction of the vitamin from neutral solutions by continuous extraction with butanol at reduced pressures. Whereas certain indophenols and indamines are comparatively stable to alkali, the vitamin indophenol is rapidly and characteristically destroyed by the addition of alkali. Mineral acid solutions of the vitamin are stable to 10 per cent sodium nitrite at 100° for 10 minutes. The vitamin can be quantitatively adsorbed by zeolite and eluted. The vitamin may be differentiated from other substances which react under the conditions of the test by the use of a borate buffer; for example,  $\beta$ -hydroxypyridine, the 4-ethoxy derivative of the vitamin, or the inner ether (I) can be differentiated from the vitamin by nature of the fact that these closely related substances will react in the presence of a borate buffer, whereas the vitamin will not. Finally, nitrogen substitution in the  $\beta$ -hydroxypyridine type of molecule gives a negative test.

Thanks are due Dr. John C. Keresztesy, with whom this work was originally undertaken, for his continued interest and advice. Thanks are also due to Mr. R. P. Buhs and Miss D. B. Hood for their cooperation.



## SUMMARY

A colorimetric method for the determination of vitamin B<sub>6</sub> with 2,6-dichloroquinonechloroimide has been described in detail. The test can be performed with 1 cc. of solution containing 0.5  $\gamma$  of the vitamin. The mechanism of the reaction has been investigated. The stability of dilute vitamin B<sub>6</sub> solutions has been studied. A variety of interfering substances has been studied and means of eliminating these have been indicated. Means of removing the vitamin from dilute solutions have been presented. A number of marks of specificity have been given. Notable among these is the use of a borate buffer. In the presence of a borate buffer, vitamin B<sub>6</sub> does not give the indophenol reaction.

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## ULTRAVIOLET ABSORPTION SPECTRUM OF PAPAIN

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In a recent paper with the above title Fruton and Lavin (1939) stated that, "The characteristic bands of tyrosine cannot be observed in the intact protein, although on acid hydrolysis appreciable amounts of tyrosine are liberated." If this statement could be confirmed, the work of Holiday (1936) on the spectrographic estimation of tyrosine and tryptophane in proteins would be of little significance. The absence of the tyrosine bands would also imply marked changes in the tyrosine component of the protein. It therefore seemed important to repeat the spectrographic investigation of papain.

### EXPERIMENTAL

Through the kindness of Dr. J. S. Fruton and Dr. A. K. Balls, two specimens of papain were obtained for study. The Fruton sample was a powder stated to contain 34.1 per cent protein, the remainder being sodium sulfate and water. The Balls sample was a suspension of papain crystals in 0.02 M sodium cyanide, containing 1.52 mg. of protein N per ml. Absorption spectra were taken on a Hilger medium quartz spectrograph No. E-316, equipped with a Spekker photometer and with a hydrogen discharge tube of my own design (Darby, 1940) as source. The wave-length scale of the spectrograph was checked against the spectrum of a mercury arc on each plate. Match points were read by eye with the aid of an enlarger.

The absorption curve obtained by me on Fruton's sample of papain (0.5 per cent solution of the powder in 0.01 N HCl, about pH 2) confirmed that of Fruton and Lavin, except that an indentation at 289 m $\mu$ , barely noticeable on their published curve, was

quite obvious on our graphs. This small extension at  $289\text{ m}\mu$  had been observed more clearly by Fruton and Lavin with another technique, and had been attributed by them to tryptophane. Use of Holiday's data for calculating, from the density of absorption at  $289\text{ m}\mu$ , the density to be expected at  $275\text{ m}\mu$ , would have shown that their curve showed altogether too much absorption in the latter region to be due to tryptophane alone. Holiday had shown

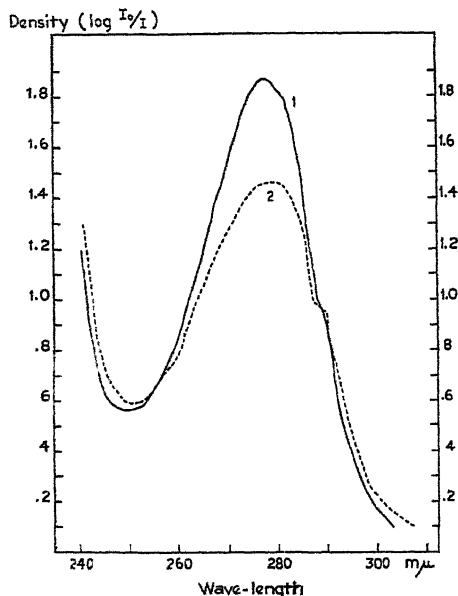


FIG. 1. Curve 1, papain (Balls) in  $0.01\text{ N HCl}$  (about pH 2); concentration  $0.138\text{ mg. of N per ml.}$ , equivalent to  $0.86\text{ mg. of protein}$ ;  $1\text{ cm. cell}$ . Curve 2, papain (Fruton) in  $0.01\text{ N HCl}$  (about pH 2); concentration  $0.121\text{ mg. of N per ml.}$ , equivalent to  $0.76\text{ mg. of protein}$ ;  $1\text{ cm. cell}$ .

that the molecular extinction coefficients of the tryptophane maxima at  $289\text{ m}\mu$  and  $275$  to  $280\text{ m}\mu$  are independent of pH over a wide range, and are therefore not liable to much alteration with change of pH when this amino acid is linked in the protein molecule.

The Balls sample, diluted to contain  $0.86\text{ mg. of protein per ml.}$ , was also investigated at pH 2 and curves of both samples are given in Fig. 1. Crystalline papain yielded a curve with better

defined structural details than are present in the curve from the amorphous material. The density relations between 289 and 275  $m\mu$  are markedly different in the two preparations, and the relatively low absorption at 250 and 300  $m\mu$  shows the Balls material to contain less absorbing contaminants than Fruton's. Calculations on the Balls curve, based on the density at 289  $m\mu$ , show that the absorption due to tryptophane at 275  $m\mu$  is being considerably reinforced by some other material which absorbs in that region. The absorption curve of tyrosine in acid solution, according to Holiday, shows a double peak at 273 and 280  $m\mu$ , while tryptophane has a single peak at 275  $m\mu$ . The absorption of these two substances in acid solution would be additive in that region.

This added absorption, if it be due to tyrosine, should be easily separable from that of tryptophane by raising the pH. Stenström and Reinhard (1925) showed that tyrosine in alkaline solution has its absorption maximum at about 290  $m\mu$ , and that the density is roughly twice as high as in acid. Also, the absorption minimum at 245  $m\mu$  in acid solution is replaced by a steep end absorption at high pH levels. These findings have been confirmed and extended by Holiday.

Solutions of crystalline papain were accordingly made up at various pH levels and absorption curves were determined. The spectrographic plates were prepared for the purpose of demonstrating shifts in the positions of maxima, rather than to bring out all the details of structure. The curves are therefore only approximate with respect to fine structure. They are shown in Fig. 2. In HCl at about pH 2, where both tyrosine and tryptophane have absorption maxima in much the same region, the primary maximum of the protein lies at 278  $m\mu$ , and the small tryptophane peak at 289  $m\mu$  is plainly discernible on the side of the curve. In 0.1 N NaOH (about pH 13) the tryptophane maximum at 280 appears as a secondary hump (Curve 1), the minimum is shifted 22  $m\mu$  towards the longer wave-lengths, and the small tryptophane peak at 289  $m\mu$  is enhanced so much by the absorption maximum at 290 of alkaline tyrosine that it now appears as the primary maximum of the protein. An intermediate pH level (12) produced an intermediate curve. The protein behaves as would be expected for a mixture of tyrosine and tryptophane.

Fruton and Lavin attempted to demonstrate the characteristic bands of tyrosine and tryptophane in various mixtures of these compounds by photographing the absorption spectra. They did not measure the densities at frequent intervals of wave-length, as was done by Holiday and by the present author. Such attempts must fail when the substances under examination absorb in the same region. Differentiation can be undertaken only when the peaks are clearly resolvable.

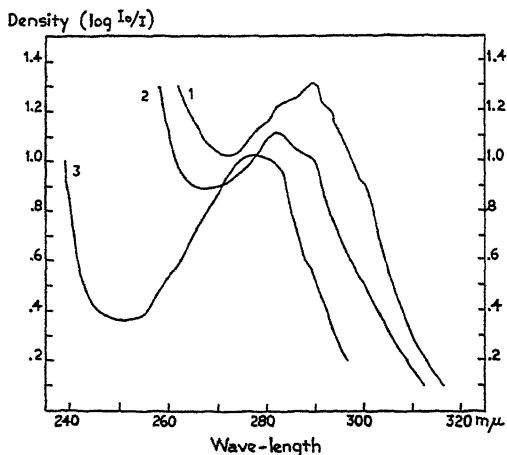


FIG. 2. Papain (Balls) at various pH levels, concentration 0.083 mg. of N per ml., equivalent to 0.51 mg. of protein; 1 cm. cell. Curve 1, in 0.1 N NaOH; Curve 2, in 0.01 N NaOH; Curve 3, in 0.01 N HCl.

The only procedure which can be used under such circumstances is to determine photometrically the entire absorption curve of the mixture and then to identify, with due regard to the relative densities, any special features of the component curves which may be distinguishable. In cases of complete superposition there is no feasible method of distinguishing the components spectrographically.

Analysis of the absorption curves obtained with crystalline papain (Balls) in this study indicates that the molecular ratio of tyrosine to tryptophane in the intact protein is not less than 4:1 (Fruton and Lavin estimated 20:3 by chemical methods).

The details of this analysis and a reexamination of the absorption curves of the aromatic amino acids will shortly be published.

#### SUMMARY

The absorption spectra of papain show the presence of both tyrosine and tryptophane in the intact molecule. The conclusions of Fruton and Lavin are shown to be invalid. The reasons for the error lie in the spectrographic technique used.

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## DISTRIBUTION AND CHARACTERIZATION OF BEEF PLASMA FATTY ACIDS\*

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Specific information regarding the distribution of fatty acids is essential to a more complete understanding of fatty acid metabolism. Such data have been obtained for a number of depot fats, but are almost entirely lacking for other tissue lipids. The present study was undertaken to supply such information for the individual types of fatty acid esters of the blood plasma. This source offers a singular combination of the different lipids, particularly in the high concentration of cholesterol esters. The latter comprises one of the least understood group of lipids. Similarity in physical properties has prevented the separation of cholesterol esters from other "neutral fat."

A method for the specific enzymatic hydrolysis of the glycerides in the presence of cholesterol esters (1) enabled us to study the fatty acids combined with glycerol and cholesterol. After enzymatic cleavage of the glycerides, the purified cholesterol esters were separated and their combined fatty acids obtained after saponification. The fatty acid composition of the cholesterol esters in plasma was shown to be unique, in that an extraordinarily large proportion (62 per cent) of linoleic acid is present, and in marked contrast to the composition of the fatty acids of the glycerides.

Cows were chosen as the experimental animals, in part because

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† National Research Council Fellow in Medicine, 1939-40.



it was desirable to obtain large quantities of material for the investigation (2). The data presented are intended to serve as a basis for the evaluation of future nutritional studies.

#### EXPERIMENTAL

*Source of Blood*—Six Holstein cows, received at the Pittsburgh branch of Armour and Company, supplied the blood used in this investigation. The animals were in good physical condition and producing milk. For 24 hours before the blood was collected the animals were fed only hay and water. Prior to their arrival at the stock-yards they had been in pasture; no seed cake had been fed to any of the animals for at least 1 month before slaughtering.

Blood was collected directly from the jugular vein after the animals were stunned. A total of 90 liters was obtained, including 1 liter of a saturated solution of potassium oxalate which was added to prevent coagulation. The oxalated blood yielded 42 liters of plasma (containing 1 liter of water added with the oxalate). No hemolysis was observed.

*Extraction of Total Lipids*—The plasma was poured slowly into 126 liters of 85 per cent alcohol with continual shaking. After 3 to 4 hours, the plasma-alcohol mixture was filtered through muslin. The alcoholic filtrate was concentrated under reduced pressure until most of the alcohol had been removed.<sup>1</sup> The concentrates from the first alcoholic extraction were extracted with petroleum ether (40-60°) in a continuous liquid-liquid extractor until the aqueous layer contained less than 15 mg. of fatty acids per 400 ml. (determined by saponification with potassium hydroxide and extraction of the liberated acids with petroleum ether). The petroleum ether extracts were reserved as a portion of the total lipid.

The remainder of the total lipid was extracted from the plasma protein with boiling ethyl alcohol in a continuous hot extractor (3). Extraction of the protein was continued until less than 50 mg. of fatty acids remained in a 100 gm. sample. The alcoholic

<sup>1</sup> Considerable foaming occurred after the volume had been reduced to about one-half. In order to concentrate the filtrates further without loss of lipid material it was necessary to connect two distilling flasks in series. Any foam arising in the first flask was broken immediately in the second flask from which distillation proceeded rapidly.

extract was concentrated at 50° and lipids were extracted from the concentrate with petroleum ether (40–60°), first in separatory funnels to remove the bulk of the material and then in the continuous liquid-liquid extractor until 400 ml. of the aqueous layer contained not more than 15 mg. of fatty acids.

The combined petroleum ether extracts were dried over anhydrous sodium sulfate. The yield of total lipid was 109.1 gm. Unextracted lipids amounted to about 2 gm.

*Separation of Phospholipids and Free Fatty Acids*—For the initial resolution, 105.5 gm. of the total lipids were poured into 4 liters of dry, freshly distilled acetone with continuous stirring. The acetone solution was allowed to stand overnight at room temperature. In the morning, 5 ml. of a saturated alcoholic solution of magnesium chloride were added and the solution was shaken and centrifuged. The supernatant liquid was decanted. The phospholipid was reprecipitated twice in 2 liters of dry acetone. The precipitate was extracted with ethyl ether and the ether solution was washed repeatedly with dilute alcohol to remove magnesium chloride. Wash waters were extracted three times with ethyl ether. The combined ether solutions were evaporated under nitrogen and the residue, after extraction with petroleum ether, yielded 14.88 gm. of phospholipid (4.0 per cent P, 66.5 per cent fatty acids).

Only a small amount of phospholipid (<0.2 per cent) remained in the combined acetone-soluble fraction which was concentrated under nitrogen and extracted with petroleum ether. In order to separate the free fatty acids, an excess of potassium hydroxide in 40 per cent alcohol was added to the petroleum ether extract and the mixture was well shaken. The petroleum ether solution was washed twice with alkaline 40 per cent alcohol, and then twice with neutral 40 per cent alcohol. The alcoholic soap solution and the wash waters were extracted in turn seven times with 1 liter portions of petroleum ether. The combined and dried petroleum ether solutions yielded 81.68 gm. of material (I No. 99.4) which represents the acetone-soluble fraction minus free fatty acids. The term "neutral fat"<sup>2</sup> is reserved for this material. The alkaline

<sup>2</sup> The expression "neutral fat" has found wide usage but has not always indicated a particular lipid preparation. Glycerides are usually implied

wash waters were acidified with hydrochloric acid and extracted with three 1 liter portions of petroleum ether. After the ether solution was washed until all hydrochloric acid had been removed and then dried over drierite, a yield of 11.50 gm. of free fatty acids (I No. 70.0, mol. wt. 300) was found.

*Enzymatic Separation of Glyceride Fatty Acids*—The “neutral fat” fraction of the blood lipids contains the free cholesterol and fatty acids esterified with both cholesterol and glycerol. Cholesterol analyses (4) of this fraction indicated the presence of 13.22 gm. of free cholesterol in the original 41 liters of plasma. In order to obtain first only those fatty acids esterified with glycerol, Kelsey’s enzymatic procedure (1) was used at this point. This method is based on the fact that a specially prepared pancreatic lipase is specific in attacking glycerides and will not catalyze the hydrolysis of cholesterol esters.

The enzyme was prepared in the following manner. 20 gm. of acetone-ether-dried hog pancreas (containing less than 0.504 gm. of extractable lipid) were stirred with 200 ml. of 0.18 N  $\text{NH}_4\text{OH}$  (final pH, 10.1) and allowed to stand overnight in the ice box. The mixture was then centrifuged 1 hour in a refrigerated angle centrifuge, filtered through gauze, and recentrifuged for 30 minutes. Acetic acid was added to 100 ml. of the decantate with careful stirring until pH 4.8 was reached. The precipitate was centrifuged, stirred in 100 ml. of water, brought to pH 9.5 with ammonia, then to pH 4.8 with acetic acid, and again centrifuged. This precipitate was stirred in 100 ml. of 5 per cent sodium chloride, brought to pH 9.5 with ammonia, then to pH 7.0 with acetic acid, and centrifuged for 1 hour. 5 per cent sodium chloride was next added to a volume of 200 ml. and ammonia was added to adjust the pH to 9.2. A yield of 35 per cent of the original lipase activity was obtained. The product had a total activity of 380 Willstätter units.

For the digestion itself, a macroadaptation of the reported pro-

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by this term and phospholipids are excluded. In differential lipid analyses the fatty acids of the neutral fat are calculated as the difference between the total fatty acids (determined) and the cholesterol ester fatty acids (calculated from cholesterol analyses). The fallacy of such a calculation is obvious, since the value obtained included glyceride fatty acids, soaps, and certain extra fatty acids.

cedure was followed with 79.64 gm. of the "neutral fat" fraction and 200 times the specified quantity of other ingredients. After 18 hours hydrolysis, an additional 50 ml. portion of the enzyme preparation was added and the reaction allowed to continue for 6 hours. No additional hydrolysis of the glycerides was observed. The mixture was then transferred to a 4 liter separatory funnel for the extraction of the fatty acids freed by lipase action. The procedure here was similar to that used for the separation of free fatty acids from the total acetone-soluble material above. Five 1 liter portions of petroleum ether were used to extract the unhydrolyzed portion from the aqueous alkaline solution containing the freed fatty acids and the ether was washed four times with 40 per cent alcohol. The glyceride fatty acids were liberated from the combined soap solutions and alcohol washings (by hydrochloric acid) and extracted with petroleum ether. A total of 10.03 gm. of fatty acids was obtained. The unhydrolyzed fraction, containing free and esterified cholesterol, weighed 69.48 gm. (calculated recovery, 99.9 per cent).

*Separation of Cholesterol Ester Fatty Acids*—The unhydrolyzed fraction remaining after the lipase digestion constituted the largest single fraction of the total blood lipids. This fraction gave values for free cholesterol corresponding to 13.04 gm. (compare with 13.22 gm. determined before lipase digestion). A portion of the unhydrolyzed lipids (55.78 gm.) was saponified with 22 gm. of potassium hydroxide in 1500 ml. of an alcohol-ether mixture (1:1). The solution was heated for 2 hours on the steam bath. Water was added to the alkaline mixture to bring the aqueous phase to a 40 per cent alcohol concentration and the solution was extracted with four 1 liter portions of a petroleum ether-ethyl ether-chloroform mixture (6:3:1). The extracts were washed three times with alkaline 40 per cent alcohol to remove any soaps and finally with neutral 40 per cent alcohol. Fatty acids were obtained from these alkaline solutions after acidification with hydrochloric acid. The unsaponifiable portion was again saponified with 10 gm. of potassium hydroxide in 1 liter of alcohol-ether (1:1) and the fatty acids and unsaponifiable matter extracted as described above. The combined yield of cholesterol ester fatty acids from the two saponifications was 20.81 gm. The total unsaponifiable material amounted to 34.70 gm. of which 88.4 per cent was cholesterol by

analysis (4). On the basis of 41 liters of plasma this corresponds to 39.54 gm. of cholesterol. An investigation of the nature of the unsaponifiable material other than cholesterol is being undertaken.

The values for the individual fractions are recorded in Table I, calculated on the basis of the total lipid in 41 liters of plasma and, for comparison, in the usual fashion as mg. per cent.

*Fatty Acid Analyses*—The free fatty acids isolated from the total acetone-soluble lipids, the glyceride fatty acids liberated by enzyme action, and the acids finally obtained after saponification

TABLE I  
*Distribution of Plasma Lipids*

Lipid fraction	Amount	Proportion
	mg. per 100 ml. plasma	per cent
Total lipid . . . . .	266.3	100.0
Phospholipid . . . . .	36.7	13.8
Acetone-soluble lipids . . . . .	229.6	86.3
Free fatty acids . . . . .	28.4	10.7
Neutral fat. . . . .	201.2	75.6
Glyceride fatty acids . . . . .	24.1	9.1
Unhydrolyzed fraction. . . . .	175.6	66.0
Cholesterol ester fatty acids . . . . .	65.5	24.6
Unsaponifiable . . . . .	108.9	41.0
Total cholesterol . . . . .	96.4	36.2
Ester " . . . . .	64.6	24.3
Free " . . . . .	31.8	11.9

of the cholesterol esters were subjected to further analysis. An initial resolution was made of each of the three groups of acids into predominantly saturated and unsaturated groups by the lead salt-alcohol procedure (5, 6). In the case of the cholesterol ester fatty acids, whose lead salts were soluble in alcohol, this was followed by a lithium salt-acetone separation (7) for the purpose of removing the extremely unsaturated acids (and also any contaminating unsaponifiable matter) which were present. The results of the separations are recorded in Table II.

Methyl esters of each separated fatty acid mixture were prepared for fractional distillation and subsequent analysis by the

same procedures already reported (6, 8). The distillation and analyses of two ester mixtures from (a) the cholesterol ester fatty acids and (b) the glyceride fatty acids are shown in Table II. The

TABLE II  
*Analyses of Methyl Ester Mixtures*

Methyl esters of cholesterol ester fatty acids (20.81 gm. acids used)					Methyl esters of glyceride fatty acids (10.03 gm. acids used)				
Acid group	Methyl ester Fraction No.	Weight	Saponification equivalent	I No. (Wijs)	Acid group	Methyl ester Fraction No.	Weight	Saponification equivalent	I No. (Wijs)
		gm.					gm.		
Pb salt	L1	0.900	269.3	90.4	Pb salt	L1	0.471	268.5	74.6
liquids,	L2	0.966	278.2	95.6	liquids	L2	0.752	279.8	95.5
Li salt	L3	1.267	283.4	122.4	(5.64	L3	0.614	282.4	104.9
solids	L4	2.083	287.6	148.4	gm.)	L4	0.443	286.5	115.6
(15.94	L5	1.436	293.2	168.6		L5	0.824	290.3	125.9
gm., I	L6	2.093	293.8	175.5		L6	0.499	294.5	132.8
No. 162)	L7	2.895	294.2	180.0*		L7	0.234	295.2	116.1
	L8	0.936	295.3	192.6		L8	1.296	325	152.3
	L9	1.281	317	191.1		Total	5.133	(302)†	(133)†
	Total	13.857	(301)	(190.3)†	Pb salt	S1	0.569	272.3	0.3
Pb salt	S1	0.448	273.3	6.0	solids	S2	0.653	274.6	0.3
solids	S2	0.239	298.7	7.9	(4.36	S3	0.547	279.6	0.3
(1.32	S3	0.446	301.4	14.3	gm.)	S4	0.397	283.7	0.3
gm., I	Total	1.133				S5	0.429	294.6	0.5
No. 8.0)						S6	0.449	297.0	0.6
Pb salt		3.110	304	205‡		S7	0.756	343	3.0
liquids,			(298)			Total	3.800	(301)†	
Li salt									
liquids‡									

\* Thiocyanogen value, 104; 2.035 gm. of acids yielded on bromination 0.15 gm. of ethyl ether-insoluble bromide, m.p. 185° (decomposition), and 1.43 gm. of ethyl ether-soluble, petroleum ether-insoluble bromide, m.p. 114°; composition of C<sub>18</sub> unsaturated esters by weight, oleic 10, linoleic 80, linolenic 10.

† Saponification equivalent after the removal of the unsaponifiable matter.

‡ Analyses (4) showed the presence of only traces of cholesterol.

§ Thiocyanogen value, 112; ethyl ether-insoluble bromide, m.p. 218–220° (decomposition), ≈ 10 per cent of the mixture; composition of C<sub>18</sub> unsaturated acids by weight, oleic 10, linoleic 70, linolenic 20.

calculated fatty acid compositions (Table III) are expressed on a molar basis. An additional calculation of the molar distribution

TABLE III  
*Fatty Acid Composition of Acetone-Soluble Plasma Lipids*

Acids	Free fatty acids (11.64 gm. $\approx$ 24.1 per cent)	Glyceride fatty acids (9.88 gm. $\approx$ 20.1 per cent)	Cholesterol ester fatty acids (26.86 gm. $\approx$ 55.5 per cent)	Total (48.38 gm. $\approx$ 100 per cent)
	<i>M per cent</i>	<i>M per cent</i>	<i>M per cent</i>	<i>M per cent</i>
Myristic.....	0.8	0.2		0.3
Palmitic.....	34.4	33.7	11.1	21.4
Stearic.....	5.2	22.2	3.3	7.6
Arachidic.....	2.7	0.5	0.3	0.8
Palmitoleic.....		2.6	4.2	2.8
Oleic.....	40.5	21.3	7.9	18.5
Linoleic.....	16.4	18.4	61.7	42.1
Linolenic.....			9.2	5.0
Arachidonic.....		1.1	2.3	1.5
Saturated.....	43.1	56.6	14.7	30.1
Unsaturated.....	56.9	43.4	85.3	69.9

TABLE IV  
*Fatty Acid Content of Beef Blood Fat*

Acid	Whole blood, Parry and Smith (2)	Plasma, Kelsey and Longenecker
	<i>per cent</i>	<i>per cent</i>
Myristic.....		0.4
Palmitic.....	10	24.5
Stearic.....	13	12.3
Arachidic.....	3	0.8
Palmitoleic.....		2.6
Oleic.....		17.4
Linoleic.....	26	36.5
Linolenic.....		4.2
Arachidonic.....	33	1.3
C <sub>22</sub> .....	10	

of all the acids present in the acetone-soluble lipids was made. These acids represent 82.8 per cent of the total acids found in the plasma lipids, the balance being the acids in the phospholipids.

It is unfortunate that the phospholipids could not be studied in detail in the course of this work. Analytical constants of the original acids of this fraction and of those obtained in a lead salt separation indicated, however, that there was no great apparent difference between the phospholipid fatty acids and the glyceride fatty acids which were analyzed in detail. The effect on the total fatty acid composition of including the phospholipid fatty acids on the assumption that their composition was the same as that found for the glyceride fatty acids was slight, as is indicated in Table IV.

#### DISCUSSION

A critical evaluation of Table I will show that despite the extreme care which was taken to prevent losses due to incomplete extraction at several stages in the preparative procedure the total lipid obtained was somewhat lower than might have been expected. The relative proportions of the individual lipids are in agreement with previous reports (9, 10) except for the phospholipid and free fatty acid fractions. The method employed for the preparation of the plasma lipid clearly involved a somewhat vigorous treatment which was necessarily resorted to in order to obtain, in so far as possible, all the lipid components in representative proportions and unaltered from their original state. Quite possibly, however, some changes may have been effected. The comparatively high value found for the free fatty acids may have been due to partial phospholipid decomposition during extraction, as Bloor has suggested (11).

The distribution of the total cholesterol as 33 per cent free and 67 per cent esterified is in accord with the generally accepted values (12). It is of considerable interest to observe, however, that the determined amount of esterified cholesterol in 41 liters of plasma (determined from cholesterol analyses) requires only 18.9 gm. of fatty acids (mean mol. wt. 275), whereas 26.9 gm. of acids were obtained as cholesterol ester fatty acids. In the customary differential blood lipid analyses these "extra" acids would be calculated as neutral fat fatty acids with the tacit assumption that the values so obtained would represent glyceride fatty acids.



The origin of the "extra" acids in the cholesterol ester fatty acid fraction is unknown at present. It is improbable, however, that they were derived from glycerides which had escaped enzymatic hydrolysis for the following reasons: (a) no further hydrolysis of "neutral fat" was observed upon the addition of fresh enzyme after 18 hours of reaction, (b) the observed hydrolysis would have been only 55 per cent complete, similar to Kelsey's observations with beef and dog plasma (13) but contrary to his regular finding that 98 to 100 per cent hydrolysis of glycerides (olive oil) took place with no hydrolysis of synthetic cholesterol esters, and (c) the respective fatty acid composition of the glycerides and cholesterol esters (Table III) indicates that not more than 15 per cent of the latter could be contaminated by a mixture of acids such as that representing the glycerides (stearic acid is the limiting factor).

The explanation of the "extra" fatty acids may be due to the determination of more than the true amount of free cholesterol by methods employing digitonin as the precipitant. Windaus (14) showed that cholesterol could form two types of compounds with fatty acids, (a) stable esters and (b) loose combinations of cholesterol, and the possible presence of the latter in plasma was indicated more recently (15) by the use of pyridine sulfate in the place of digitonin. Free cholesterol is found by the latter method to be only 6 to 10 per cent of the total plasma cholesterol instead of the usual one-third found by digitonin. The difference between the two is considered as loosely bound cholesterol. The "extra" fatty acids would require 10.97 gm. of the cholesterol determined as "free" by the digitonin method. This would leave 2.07 gm. as entirely uncombined cholesterol, or about 5 per cent of the total plasma cholesterol, which is in good agreement with Sobel's analyses.

In 1929, Channon and Collinson (10) reported that about 55 per cent of the total fatty acids present in the acetone-soluble portion of plasma lipids was esterified with cholesterol. From glycerol determinations, they estimated that about one-half of the "residual fatty acids" was present as glycerides. The nature of the remainder was undetermined. There is apparent excellent agreement between their results and the ones presented above. In both cases, the acids esterified with cholesterol amount to 55

per cent of the total acids of the acetone-soluble lipids, and the free fatty acids and those esterified with glycerol are 21 to 23 per cent. Such agreement seems to have more significance because of the different methods of approach in the two investigations. It may, however, be more apparent than real, for the value of 55 per cent for cholesterol ester fatty acids found in these investigations was an actual experimental value. In Channon and Collinson's work, the figure was calculated. In the data reported here, "cholesterol ester fatty acids" include the "extra" fatty acids. The latter resembled the cholesterol ester fatty acids in composition and, as esters, they were resistant to lipase hydrolysis. In Channon and Collinson's studies, however, such "extra" fatty acids, which may really be loosely bound to cholesterol as discussed above (or to some unsaponifiable material other than cholesterol), were calculated with the 45 per cent "residual acids" and therefore included as either glyceride fatty acids, free fatty acids, or soaps.

One striking observation from these studies of the acid composition of the separated groups of plasma lipids is the marked unsaturation of the cholesterol esters. Such an observation is not new. Bloor (9, 11, 16) suggested that unsaturated cholesterol esters were present in plasma either as the linoleate or as a mixture of the oleate with esters of acids more unsaturated than oleic acid. Channon and Collinson (10) concluded, however, that the evidence was too meager to support a reliable deduction as to the unsaturation of acids coupled with cholesterol. In each of these investigations, the iodine values of the fatty acids of the acetone-soluble fraction were considerably higher than the values for the phospholipid fatty acids. Bloor *et al.* (17) made a similar observation more recently.

The data presented in Tables II and III show beyond any reasonable doubt the decided unsaturation of the cholesterol ester fatty acids. The predominance of a single fatty acid, linoleic acid, has considerable interest. Its occurrence here can scarcely be regarded as fortuitous; neither can it be accounted for by absorption from a dietary supply, since the animals received little, if any, of the acid in food. It is the more unusual to find such a large amount (42 per cent of all the acids of the acetone-soluble lipids, or 36 per cent of all the plasma acids) in an animal which

customarily deposits only small amounts of linoleic acid in its body fat.

The presence of such a large amount of unsaturated fatty acid in the free state would probably be toxic owing to its hemolytic properties (18). Cholesterol esterification serves to reduce the hemolytic action. At the same time, cholesterol is converted into a form suitable for transport. Bloor (19) and Sperry and Brand (20) have recently collected the evidence relating cholesterol with fatty acid transportation mechanisms.

A calculation of the fatty acid composition of all the acetone-soluble lipids was made on the basis of the actual proportions of the fatty acids obtained. These data are included in Table III. In addition to these 48.38 gm. of acids from the acetone-soluble lipids, there were 10.00 gm. of acids from the phospholipids. Since the saponification equivalent, iodine value, and thiocyanogen value were nearly the same for the phospholipid fatty acids as determined for the glyceride fatty acids, it may be assumed, for the calculation, that the two have the same composition. On this basis, the composition of the total mixture of acids which would have been obtained by hydrolysis of the total plasma lipid was calculated. The figures are compared in Table IV with Parry and Smith's data for the fatty acid composition of whole ox blood (2).

The considerable discrepancy in the two analyses is not readily explained. Parry and Smith found so little linoleic acid present in their samples that they were led to conclude, "This finding confirms the view that the more unsaturated acids of the diet are those which are most readily and quickly oxidized by the body and may well be connected with the fact that these particular acids are 'essential' in the diet." On the other hand, they report the occurrence of large amounts of highly unsaturated  $C_{20}$  and  $C_{22}$  acids. The presence of the latter was demonstrated by the preparation of bromides whose elementary composition agreed well with that of the octabromo derivative of arachidonic acid. This fact lends good support to the calculations Parry and Smith made for the composition of their higher boiling unsaturated ester fractions. It should be recognized, however, that the presence of any unsaponifiable material in such ester fractions would raise the

equivalent weight and have the over-all effect of increasing the calculated amount of the  $C_{20}$  and  $C_{22}$  acids.

Parry and Smith used whole blood for their analyses rather than plasma. They point out the fact that by their saponification procedure they obtained only about 70 per cent of the expected total fatty acids. It seems reasonable to believe that they may not have saponified completely the cholesterol ester portion and thereby missed finding the quantities of linoleic acid which we have determined to be present (especially in that fraction) and which others have found consistently (9-11, 16, 17, 19).

#### SUMMARY

The total lipids from 41 liters of beef plasma were resolved into four fractions for a quantitative study of their component fatty acids. Phospholipids (I) were obtained by acetone precipitation, free fatty acids (II) after ether extraction of an alkaline aqueous suspension of the acetone-soluble portion, glyceride fatty acids (III) by specific enzyme hydrolysis, and cholesterol ester fatty acids (IV) by saponification of the remaining ester fraction not attacked by the purified pancreatic lipase. The fatty acid content of this fraction was higher than that calculated from the ester cholesterol content, showing the presence in this fraction of fatty acid compounds other than glycerol or cholesterol esters.

Ester distillation analyses of these acids showed the presence in the total acetone-soluble lipids of 42 per cent linoleic acid, 21 per cent palmitic acid, and 18 per cent oleic acid, with lesser amounts of stearic, arachidic, palmitoleic, linolenic, and arachidonic acids. The saturated acids were found almost entirely in Fractions I, II, and III, whereas in Fraction IV, the acids were 85 per cent unsaturated and contained an unusually high amount (62 per cent) of linoleic acid which was isolated in quantity as tetrabromostearic acid.

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## STUDIES ON THE CHEMISTRY OF BLOOD COAGULATION

### XIII. THE PHOSPHATIDE CONSTITUENTS OF THE THROMBOPLASTIC PROTEIN FROM LUNGS\*

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The isolation from lungs and the properties of the thromboplastic protein, *i.e.* the agent which, together with calcium, effects the conversion of prothrombin to thrombin, were discussed in a previous paper from this laboratory (1). Simultaneously, it was shown (2) that this compound, which is a lipoprotein, was disrupted by heparin in such a manner as to yield an anticoagulant heparin-protein complex together with free phosphatides.

The thromboplastic factor plays a pivotal part in the initiation of blood clotting under physiological conditions. It is probable that this function is connected with the availability of the phospholipids contained in the thromboplastin, since the phosphatides from animal tissues themselves are known to have marked thromboplastic properties. It appeared, for this reason, of interest to study the composition of the phosphatides carried in the thromboplastic protein.

This protein, when obtained by isoelectric precipitation, in addition to the firmly bound phosphatides carries with it a considerable amount of steroids, steroid esters, and probably also neutral glycerides. These substances can be removed by treatment of the protein with acetone. The phosphatides which form

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the subject of the present communication were isolated from the lipoprotein by extraction with alcohol-ether mixtures. They were separated into an ether-insoluble fraction from which sphingomyelin was isolated and an ether-soluble portion which in turn was subdivided into the alcohol-soluble "lecithin" and the alcohol-insoluble "cephalin" fractions. Both the "lecithin" and "cephalin" showed a remarkably strong thromboplastic potency almost comparable to the very active phosphatide preparations isolated from blood platelets in this laboratory (3). To speak of lecithin and cephalin, at least in material isolated from organs other than the central nervous system, is, of course, entirely a matter of convention, since the separation of the individual phosphatides based on their differential solubility in alcohol is unsatisfactory. The difficulties inherent in this method will be exemplified in a study of heart phosphatides which, it is hoped, will be presented at a later date.

The study of the composition of the two fractions which exhibited thromboplastic activity was somewhat handicapped by scarcity of material. The mixture of saturated acids isolated in both cases consisted mainly of palmitic and stearic acids. The liquid acids, in addition to small amounts of more highly unsaturated compounds, consisted for a large part of acids with one double bond. These apparently contained oleic acid together with compounds of higher molecular weight, as shown by the hydrogenation experiments reported. The glycerophosphoric acid isolated was a mixture of about 4 parts of the  $\alpha$  and 1 part of the  $\beta$  form. Choline was isolated from both fractions; but whereas in the case of the "lecithin" practically all the non-amino N occurred in the form of this base, this was not true of the "cephalin" fraction in which only 5 per cent of the non-amino N could be accounted for as choline. Both fractions yielded very small amounts of ethanolamine. It is obvious that one or more unidentified primary bases must be responsible for the major part of the amino N contained in both fractions.

In this connection a recent report (4) is of interest in which the occurrence of a hydroxyamino acid, presumably serine, in brain cephalin was shown to be probable. It has long been evident that the amounts of ethanolamine which can be isolated from tissue cephalin fractions other than brain and egg cephalin (com-

pare (5)) are much smaller than would be expected from the structure generally assigned to this phosphatide. It is entirely possible that the phosphatides discussed in the present paper contained a hydroxyamino acid or a primary amine different from ethanolamine and that the thromboplastic activity of tissue cephalin fractions is connected with the presence of these unknown substances.

The literature on lung lipids is scarce. The isolation of lecithin (6), a "phosphosulfatide" (7), lignoceryl sphingosine (8), and sphingomyelin (9) has been reported. Mills (10) considered the phosphatides of lung thromboplastin to be all cephalin.

It is remarkable that the complex phosphatide mixture here described, comprising at least three phosphatides of different electrophoretic properties, migrated uniformly when bound in the thromboplastic protein ((1) and unpublished results). Similar observations have been reported for the lipoprotein forming the stabilizing film on the fat droplets in cream (11, 12). It is not easy to visualize the type of binding between proteins and phospholipids in naturally occurring lipoproteins. The fact that phosphatides can be extracted from these complexes would seem to exclude the possibility that lipids containing a hydroxyamino acid could be built into them by way of peptide linkages. Compounds of this hypothetical type may, however, play a rôle in intermediary phospholipid metabolism.

#### EXPERIMENTAL

##### *Isolation of Phosphatides*

The phosphatides were extracted from the thromboplastic protein by the methods described in Paper IX of this series (1); *viz.*, by extraction with alcohol-ether (a) of a preparation which had previously undergone drying in a high vacuum in the frozen state and washing with acetone, or (b) of a solution of the thromboplastic protein at alkaline pH. The second method naturally yielded an extract containing considerable amounts of acetone-soluble substances (glycerides, free and esterified cholesterol) which were separated from the phosphatides by precipitation with acetone in the normal manner.

An example of the lipid yield obtained by extraction of the dry



protein will be found in the next paragraph. A representative experiment carried out according to the second extraction procedure follows. 450 cc. of a solution of the thromboplastic protein in borate buffer of pH 8.8 containing 1.13 mg. of N and 0.104 mg. of P per cc. were slowly dropped into 2 liters of boiling alcohol-ether (1:1). The mixture was kept at room temperature for 15 hours and filtered. The *protein residue* was washed with alcohol-ether and ether and dried *in vacuo* over  $P_2O_5$ . It weighed 2.88 gm. and contained N 14.5, P 0.40. The extract was evaporated *in vacuo*, and the residue dissolved in  $CHCl_3$ , filtered, and again evaporated to dryness. The total lipids weighed 3.06 gm. (51.5 per cent of the thromboplastic protein). A cholesterol determination, for which we are indebted to Dr. W. M. Sperry of the New York State Psychiatric Institute, showed the presence in this material of 6.3 per cent free *cholesterol* and 0.7 per cent *cholesterol esters*. A portion of the lipids (2.76 gm.) was dissolved in  $CHCl_3$  and precipitated with acetone. The *acetone-insoluble fraction* weighed 0.88 gm. (16.5 per cent of the thromboplastic protein, 31.9 per cent of the total extracted lipids).

#### *Fractionation of Phosphatides*

A preparation of the thromboplastic protein which had been dried in the frozen state in a high vacuum and exhaustively washed with acetone (P 0.92), weighing 8.00 gm., was extracted with two portions of 200 cc. of alcohol-ether (1:1) for 7 days. During that period the mixture was intermittently refluxed every day for a total of 60 hours. The filtered extraction residue weighed 6.50 gm. (P 0.38).

The extract was evaporated *in vacuo* and the residue dissolved in warm ether. Prolonged cooling of this solution resulted in a precipitate which was centrifuged off and washed with chilled ether. It then was dissolved in  $CHCl_3$  and precipitated from its filtered solution by the addition of 3 volumes of acetone: *Fraction 1*, 551.6 mg. of a white powder (compare Table I). A portion of this material (456.6 mg.) was dissolved in 3 cc. of a mixture of 5 parts of ligroin (b.p. 70–90°) and 1 part of absolute alcohol. The addition of 9 cc. of absolute alcohol produced a precipitate which, after being chilled, was centrifuged off, washed with chilled alcohol, and dried, giving *Fraction 1a*, 72.3 mg. of a white powder. The

combined supernatant and washings were evaporated under  $N_2$ . The residue was dissolved in hot ethyl acetate from which it separated on cooling as *Fraction 1b*, 339.9 mg. of a white powder.

The combined ether supernatants of Fraction 1 were concentrated. The addition of 3 volumes of acetone yielded, after being chilled, *Fraction 2*, 446.3 mg. of a brown paste. The supernatant of Fraction 2, evaporated to dryness and precipitated with acetone from a small amount of ether, yielded *Fraction 3*, weighing 306.8 mg. The dry residue resulting from the evaporation of the supernatant of Fraction 3 formed *Fraction 4*, weighing 157.3 mg. The total lipids extracted amounted to 1.4620 gm. (18.3 per cent

TABLE I  
*Phosphatide Fractions from Thromboplastic Protein*

Fraction No.	P	N	NH <sub>2</sub> -N	Iodine value	Plasma clotting time*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>min.</i>
1	3.3	2.2		17.9	
1a	2.7	1.9			64
1b	3.9	2.6	0.9	16.2	94
2	3.5	2.1	1.3	45.7	15
3	2.9	2.1	1.7	56.1	18
4	1.7	1.8	1.8	68.5	24

\* The control clotting time of the chicken plasma used was 175 minutes. The concentration of the lipid emulsions was 2 mg. per cc., except in Fraction 1a which was tested in a concentration of 1 mg. per cc.

of the thromboplastic protein). The analytical data are summarized in Table I. The determination of the iodine values was carried out according to Rosenmund and Kuhnhehn (13). The clotting activity was assayed by the method described previously (3) with chicken plasma as substrate.

The preceding experiments had shown that the clotting activity of the phospholipids resided in the material which was soluble in ether and insoluble in acetone. For the purpose of identifying the constituents of these phosphatides the extracts obtained from several preparations of the thromboplastic protein (corresponding to a total of 13 kilos of wet beef lungs) were pooled and divided into three main acetone-insoluble fractions: (a) the material insoluble in ether, soluble in alcohol, weighing 1.65 gm.;

(b) the material soluble in ether and in alcohol ("*lecithin*"), weighing 1.27 gm.; (c) the material soluble in ether, insoluble in alcohol ("*cephalin*"), weighing 0.96 gm. The analytical figures are given in Table II.

The "*lecithin*" fraction was precipitated from its alcoholic solution as the  $\text{CdCl}_2$  double salt. This compound, in contrast to the one normally obtained, was completely soluble in cold ether (compare (14)). The ether solution was evaporated, and the residue dissolved in  $\text{CHCl}_3$  and freed of Cd by passing dry ammonia through the solution. The "*lecithin*" fraction was recovered from the concentrated filtrate by precipitation with 4 volumes of acetone.

TABLE II  
*Main Phosphatide Fractions*

Fraction	P	N	$\text{NH}_2\text{-N}$	Iodine value	Plasma clotting time*
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>min.</i>
Alcohol-soluble (" <i>lecithin</i> ")	3.1	2.0	1.3	46.9	15
Alcohol-insoluble (" <i>cephalin</i> ")	2.8	2.2	1.4	55.9	21
Sphingomyelin	3.7	3.6		16.4	

\* The control clotting time of the chicken plasma used was 60 minutes. The concentration of the lipid emulsions was 2 mg. per cc.

The total of the material insoluble in ether, soluble in ligroin-alcohol (1.65 gm.), corresponding to Fraction 1b in Table I was treated with Reinecke salt according to the method of Thannhauser and collaborators (15). The resulting precipitate, after being washed with cold methyl alcohol, ether, and acetone, weighed 1240 mg., corresponding to 977 mg. of sphingomyelin. The reineckate was decomposed with silver nitrate and the resulting crude *sphingomyelin* (860 mg.) purified by treatment of its solution in hot alcohol with norit and repeated precipitation from  $\text{CHCl}_3$  with acetone. The final product weighed 130 mg. and formed a white powder. Analytical data will be found in Table II.

#### *Composition of Phosphatides*

*Hydrolysis*—The hydrolysis was carried out with 1232 mg. of the "*lecithin*" and 880 mg. of the "*cephalin*" fractions. Each

phosphatide was refluxed in 35 cc. of absolute methyl alcohol containing 5 per cent of dry HCl for 12 hours in an N<sub>2</sub> atmosphere.<sup>1</sup> The resulting *methyl esters of the fatty acids* were separated from the water-soluble constituents in the usual manner. Small amounts of free fatty acids removed from the esters (54.4 mg. from the "lecithin," 40.4 mg. from the "cephalin") were reesterified and added to the main fractions. The yields are recorded in Table III.

*Solid Fatty Acids*—The methyl esters were saponified and the free fatty acids recovered from the reaction mixtures in the yields indicated in Table III. The acids were separated into the solid and liquid fractions by the lead salt-alcohol procedure. From the "lecithin" fraction 400.7 mg. of solid acids and 284.4 mg. of liquid acids were obtained; in the case of the "cephalin" fraction the yields were 220.4 mg. of solid and 206.5 mg. of liquid acids.

TABLE III  
*Yields of Hydrolysis Products from Phosphatides*

Fraction	"Lecithin"	"Cephalin"
	mg.	mg.
Phosphatide hydrolyzed. . . . .	1232.0	880.0
Fatty acid methyl esters. . . . .	757.9	516.2
" acids. . . . .	700.7	448.1
Ba glycerophosphate. . . . .	342.0	166.0
Choline chloride-6HgCl <sub>2</sub> . . . . .	943.2	47.1
Ethanolamine picrolonate. . . . .	32	9

Repetition of the lead salt-alcohol separation with both solid acid fractions increased the amounts of *liquid acids* isolated to 333.8 mg. from the "lecithin" and 235.3 mg. from the "cephalin."

Recrystallization from methyl alcohol and acetone of the *solid acids* isolated from the "lecithin" led to complex mixtures. A portion of the acids was, therefore, converted into the methyl esters and 235.6 mg. of the esters were subjected to fractionation in a high vacuum in a four bulb tube in the arrangement described in previous publications (16). The main fraction (Fraction A) distilled over the temperature range of 83–92° at a pressure of 10<sup>-3</sup> mm. of Hg and formed 162.2 mg. of a colorless oil. A second small fraction (Fraction B), 23.9 mg. of a crystalline solid, dis-

<sup>1</sup> All operations, as far as possible, were carried out in an inert gas atmosphere.

tilled around  $110^{\circ}$ . The distillation residue was an orange-colored oil. After saponification 156 mg. of Fraction A and 21 mg. of Fraction B were obtained. Fraction A, after two recrystallizations from methyl alcohol, yielded 49.1 mg. of white crystals which melted at  $59-60^{\circ}$  and had a molecular weight (by titration) of 273.<sup>2</sup> Recrystallization of Fraction B yielded 9.3 mg. of crystalline material which melted at  $62.5-63.5^{\circ}$ . The melting point on admixture of pure palmitic acid was  $61.5-62.5^{\circ}$ , the molecular weight 272. Both fractions obviously represented mixtures of about equal amounts of *palmitic* and *stearic acids*.

Because of the small amounts of *solid acids* from the "*cephalin*" available no fractional distillation was carried out. The acids were twice crystallized from methyl alcohol, once from acetone, and finally again from methyl alcohol. The resulting crystalline material weighed 34 mg. It melted at  $62-63^{\circ}$  and had a molecular weight of 305.5. The combined mother liquors were concentrated to dryness, the residue precipitated as Pb salt from an alcoholic solution, and the acids recovered from the Pb salts were crystallized from methyl alcohol. The resulting material (62 mg.) melted at  $58-60^{\circ}$  and had a molecular weight of 274.

*Liquid Fatty Acids*—The unsaturated acids were fractionated as bromine addition products according to the method of Miura (17). To a solution of the acids in ether at  $-10^{\circ}$  a slight excess (as calculated from the iodine values of the phosphatides) of an ethereal bromine solution (1 cc. of bromine in 20 cc. of ether) was added. After being cooled overnight the mixtures were concentrated to dryness under  $N_2$  and the residues, after being dried *in vacuo*, treated with 20 cc. of warm chloroform. The mixtures were centrifuged and the insoluble *polybromides* washed with two 5 cc. portions of chloroform. The supernatants were evaporated and the residues taken up in 5 cc. of carbon tetrachloride. The insoluble *hexabromides* were centrifuged off and washed with 5 cc. of carbon tetrachloride. The supernatants were evaporated and the residues treated with 30 cc. of petroleum ether (b.p.  $30-60^{\circ}$ ). Centrifugation at  $-10^{\circ}$  removed the insoluble *tetrabromides*. By evaporation of the supernatants the  *dibromides* which formed

<sup>2</sup> All microtitrations were carried out with 0.03 N alcoholic KOH, standardized by means of pure palmitic acid, and phenolphthalein as indicator.

the bulk of the bromination products were obtained. The weights of the various brominated fractions are given in Table IV.

The debromination of the dibromides with Zn in glacial acetic acid yielded the *debrominated acids*, 284 mg. from the "lecithin," 200 mg. from the "cephalin." These substances were dissolved in glacial acetic acid and hydrogenated by means of the platinum oxide catalyst (18). The amount of hydrogen absorbed was 84 per cent of the theoretical amount (calculated for oleic acid) in the case of the "lecithin" fraction, and 75 per cent in the case of the "cephalin" fraction. The weights of the *hydrogenated acids* which were white solids were 277 mg. from the "lecithin," 183 mg. from the "cephalin." The acids were first purified as the alcohol-insoluble lead salts and then recrystallized from methyl alcohol. The material from "lecithin" melted at 62–63° and had

TABLE IV  
*Fractionation of Unsaturated Fatty Acids*

Fraction	"Lecithin"	"Cephalin"
	mg.	mg.
Polybromides. ....	3.0	3.0
Hexabromides.....	3.5	0.7
Tetrabromides....	52.4	52.6
Dibromides.....	486.0	298.6

a molecular weight of 292; the one from "cephalin" had a melting point of 62–62.5° and a molecular weight of 318. Admixture of pure palmitic acid in both cases produced a depression of the melting point of about 5°. These acids, even after repeated recrystallization from methyl alcohol, still had comparatively high molecular weights; *viz.*, 298 and 301 for the "lecithin" and "cephalin" fractions respectively.

*Glycerophosphoric Acid*—The aqueous solutions, remaining after the extraction of the fatty acid methyl esters by means of ether, as described above, were just neutralized to Congo red with silver carbonate. The mixtures were filtered through celite, the filtrates treated with H<sub>2</sub>S, aerated, and again filtered. Inorganic phosphate was removed by the addition of Ba(OH)<sub>2</sub> until the solutions were slightly alkaline to phenolphthalein. After centrifugation the crude barium glycerophosphate fractions were precipitated

from the supernatants by means of 2 volumes of alcohol. The precipitates were redissolved in water, treated with  $\text{CO}_2$ , the mixtures centrifuged, and the barium salts recovered by precipitation with alcohol. This procedure was repeated once more, when *barium glycerophosphate* was obtained in the amounts indicated in Table III.

<i>Analysis</i> — $\text{C}_3\text{H}_7\text{O}_5\text{PBa}$ .	Calculated.	P 10.1, Ba 44.7
307.5	Found (from "lecithin").	" 10.2, " 44.3
	" ( " "cephalin").	" 9.7, " 44.8

The yields in the case of "lecithin" were 73 per cent and in the case of "cephalin" 51 per cent of those calculated for a stearyl-oleyl phosphatide.

In both fractions the distribution of the  $\alpha$  and  $\beta$  forms was determined according to Karrer and Salomon (19). From 321 mg. of the "lecithin" *glycerophosphate* 35 mg. of the  $\text{Ba}(\text{NO}_3)_2$  double salt of the  $\beta$  form were isolated in a total volume of 8 cc. This corresponds to a total of 99 mg. of  $(\text{C}_3\text{H}_7\text{O}_5\text{PBa})_2 \cdot \text{Ba}(\text{NO}_3)_2$ , if the solubility of the complex (8 mg. per cc.) is taken into account (19); i.e., 22 per cent of the "lecithin" *glycerophosphoric acid* was the  $\beta$  form. The filtrate on being heated deposited 176 mg. of *barium  $\alpha$ -glycerophosphate*. The analysis of the "cephalin" *glycerophosphate* indicated the presence of 23 per cent of the  $\beta$  form.

*Bases*<sup>3</sup>—The alcohol-water mother liquors from the precipitation of the barium *glycerophosphate* fractions were acidified with HCl and evaporated to dryness *in vacuo*. The solutions of the residues in hot alcohol were filtered and again evaporated. This procedure was repeated once more. To the solution of the remaining material in 25 cc. of alcohol the same volume of a saturated alcoholic  $\text{HgCl}_2$  solution was added. The dense white precipitates were washed with alcohol and dried. The weights of the *choline chloride-6HgCl<sub>2</sub> double salts* (20) were 943.2 mg. from "lecithin" (corresponding to 87 per cent of the non-amino N contained in the phosphatide) and 47.1 mg. from "cephalin" (corresponding to 5 per cent of the non-amino N contained in the phosphatide). The

<sup>3</sup> The effectiveness of the separation method here employed has recently been checked in this laboratory by Dr. DeWitt Stetten, Jr., with compounds containing the  $\text{N}^{15}$  isotope. We wish to thank Dr. Stetten for communicating to us his unpublished results.

substances were for analysis recrystallized from water as white needles.

<i>Analysis</i> — $C_5H_{14}NOCl \cdot 6HgCl_2$ .	Calculated. N 0.79
1768.7	Found (from "lecithin"). N 0.83
	" ( " "cephalin"). " 0.79

Both fractions melted with decomposition at 241–242°. The melting points reported in the literature ranged from 242° to 251° (20).

The alcoholic supernatants from the choline chloride double salt were freed of Hg by means of  $H_2S$  and the filtrates evaporated to dryness. The residues, dissolved in 1 cc. of water, were adsorbed on CaO, freshly prepared by heating  $Ca(OH)_2$  at 800° for 17 hours. The mixtures were extracted in a Soxhlet apparatus with 150 cc. of a saturated solution of picrolonic acid in ether (21). The resulting yellow needles were filtered off and washed with ether containing 12 per cent of alcohol, until the washings were colorless. The weights of *ethanolamine picrolonate* were 32 mg. from "lecithin," m.p. (with decomposition) 225–226° (corresponding to 9 per cent of the amino N contained in the phosphatide), and 9 mg. from "cephalin," m.p. (with decomposition) 223–224° (corresponding to 3 per cent of the amino N contained in the phosphatide).

#### SUMMARY

The phosphatides, firmly bound in the thromboplastic protein from lungs, were extracted from the protein by means of alcohol-ether and fractionated. Sphingomyelin was purified as the reineckate. Both the "lecithin" and "cephalin" fractions showed considerable clotting activity which was approximately equal in both fractions. The active substances were hydrolyzed and characterized by their content of fatty acids, glycerophosphoric acid (both the  $\alpha$  and  $\beta$  forms), and bases. The saturated acids were mainly a mixture of palmitic and stearic acids. The unsaturated acids were fractionated by means of the bromine addition products, the bulk of which consisted of dibromides. Most of the non-amino N of the "lecithin" was isolated as choline. Ethanolamine, determined as the picrolonate, accounted for only 3 and 9 per cent of the amino N of the "cephalin" and "lecithin" fractions



respectively. The presence of another primary base, possibly connected with the thromboplastic activity of the phospholipids, is suggested.

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# THE DETERMINATION OF CYSTINE: THE USE OF CUPROUS OXIDE FOR SIMULTANEOUS REDUCTION AND PRECIPITATION OF CYSTINE AS THE CUPROUS MERCAPTIDE\*

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The determination of cystine in protein hydrolysates is made difficult by the presence of other amino acids and colored decomposition products. The most satisfactory method (1) available for avoiding such interference is to reduce the cystine, precipitate the resulting cysteine as the cuprous mercaptide, and subsequently determine the cysteine in the precipitate. Recently this method has been modified for the determination of small amounts of cystine (2). In these methods (1, 2) the cystine was reduced with tin and zinc, respectively, prior to precipitation with cuprous oxide. We found that this preliminary reduction is unnecessary and that the addition of cuprous oxide to an acid solution will both reduce and precipitate the cystine quantitatively. Cuprous chloride has previously been used (3) in this manner, and, very recently, since our method was developed, a similar direct use of cuprous oxide has been reported (4) for the isolation of cystine from protein hydrolysates.

The cuprous oxide analytical method has been shortened by omitting the use of an extraneous reducing agent such as zinc. Furthermore the usefulness of this method has been broadened to permit the analysis of material containing nucleic acid. Zinc was not only found unnecessary in our studies but its presence gave a troublesome precipitate with the phosphate resulting from

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the hydrolysis of the nucleic acid<sup>1</sup> in the material being analyzed. This precipitate, probably zinc phosphate, may have adsorbed some cysteine which was not subsequently precipitated as the mercaptide. When zinc was not used, the results were regular and satisfactory. Thus by the direct use of cuprous oxide, quantitative precipitation of cysteine could be obtained in the presence of nucleic acid. However, the nitrogen content (2) of such precipitates cannot be used to estimate the cysteine present, for high cysteine values were obtained when this was done. This is probably due to the precipitation of purines (5) not destroyed by hydrolysis. We have determined the cysteine in the mercaptide precipitates by the gravimetric estimation of the sulfur, after oxidation in the peroxide bomb, and by the Sullivan colorimetric method after removal of the copper with thiocyanate. The latter method is recommended because of its specificity, the small size of the sample required, and the shorter time necessary for the analysis.

#### EXPERIMENTAL

*Precipitation of Cysteine with Cuprous Oxide*—Material sufficient to give about 24 mg. of cysteine<sup>2</sup> was hydrolyzed by refluxing 18 hours with 50 cc. of 1:1 hydrochloric acid. The hydrolysate was concentrated to complete dryness *in vacuo*,<sup>3</sup> a boiling water bath being used toward the end. The residue was taken up in distilled water and 2 cc. of N hydrochloric acid were added to produce the necessary acidity for the precipitation. This solution was filtered into a 100 cc. volumetric flask and diluted to the mark. 30 cc. aliquots were taken for the mercaptide precipitation when a gravimetric sulfur determination was made and 10 cc. aliquots

<sup>1</sup> Dried, lipid-free bull spermatozoa, one of the materials studied, contain 27 per cent nucleic acid which produces 8.5 per cent of phosphoric acid on hydrolysis. The heads of bull spermatozoa contain 40 per cent nucleic acid.

<sup>2</sup> With our preparations of bull spermatozoa about 0.5 gm. was required.

<sup>3</sup> Graff *et al.* (2) recommended that the HCl be "largely removed," but we found the required acidity was obtained more conveniently by taking to complete dryness and adding the specified amount of N HCl. Also in subsequent adjustment of the pH with sodium acetate it was found by trial that the addition of the specified amount of sodium acetate would give the required pH and the use of an indicator was not necessary.

when the Sullivan method was employed. The aliquot in a 50 cc. centrifuge tube was heated to boiling and about 8 times the theoretical amount of cuprous oxide suspension<sup>4</sup> was added with stirring. As the cuprous oxide was added, it went into solution; then the greenish gray mercaptide precipitate began to form. About a minute after the last of the reagent was added, 0.5 cc. of a sodium acetate solution (110 gm. of the trihydrate in 100 cc. of water) was introduced with stirring. This brought the pH to about 4.0, the point of minimum solubility of the mercaptide, and the precipitate flocculated. After standing for 40 minutes at room temperature for complete precipitation, the solution was centrifuged 5 minutes, and the clear, supernatant fluid carefully decanted. The mercaptide was washed<sup>5</sup> once by suspending in 25 cc. of 95 per cent ethyl alcohol for several minutes, then centrifuged again, and decanted. In the subsequent procedure either gravimetric sulfur or colorimetric cysteine determinations were made.

*Gravimetric Sulfur Determination*—The alcohol-washed precipitate of the 30 cc. aliquot was dried in the centrifuge tube at 100° for 15 minutes. The bulk of it was removed to a Parr peroxide bomb cup and the remainder transferred by the use of repeated small portions of the fusion accelerator (600 mg. of potassium perchlorate plus 100 mg. of benzoic acid). The fusion was made, the melt dissolved, and barium sulfate precipitated according to a recommended method.<sup>6</sup> The precipitate of barium sulfate was

<sup>4</sup> We have found the following procedure to give a satisfactory cuprous oxide suspension: dissolve 7 gm. of copper sulfate pentahydrate in 100 cc. of water and add to 100 cc. of a solution made of 170 gm. of sodium tartrate dihydrate, 520 cc. of water, and 80 cc. of a saturated solution of sodium hydroxide. Heat the mixture to boiling and add a solution of 1 gm. of glucose in 200 cc. of water. Continue the boiling several minutes, then wash the precipitate with hot water by decantation until the washings are neutral (about four times), and finally suspend in 100 cc. of water for use. This suspension contains about 25 mg. of cuprous oxide per cc.

<sup>5</sup> Graff *et al.* (2) washed it three times with citrate buffer. However, this was done in order to remove contaminating nitrogenous compounds, as nitrogen was estimated as a measure of the cysteine. The alcohol was found satisfactory and facilitated drying.

<sup>6</sup> The use of the several reagents in the precipitation of the fusion is discussed in the manual accompanying the Parr bomb.

filtered on a previously ignited and weighed filter crucible having suitable porosity<sup>7</sup> and ignited 1 hour at a dull red heat in the electric furnace. From the weight of barium sulfate, the cystine content of the material was calculated. Some results obtained with cystine, bull spermatozoa, and horse serum albumin are given in Table I.

TABLE I

*Cystine Sulfur Recovered in Cuprous Mercaptide Precipitates by Gravimetric and Sullivan Methods*

Method	Material	Dry weight of sample	Cystine sulfur found
		mg.	per cent
Gravimetric	Cystine	10.5	25.9 $\pm$ 0.5*
	Spermatozoa†	246	1.10 $\pm$ 0.02
	" (10 mg. cystine added)	211	1.11 $\pm$ 0.02‡
	Serum albumin§	114	1.52 $\pm$ 0.08
Sullivan	Cystine	0.80	25.4 $\pm$ 1
	Spermatozoa	19.5	0.96 $\pm$ 0.06
	Serum albumin	12.7	1.57 $\pm$ 0.04

\* The dried cystine used contained  $25.9 \pm 0.4$  per cent sulfur by direct estimation (corrected for 0.9 per cent ash). The theoretical sulfur content of cystine is 26.7 per cent.

† The total sulfur content of this dried, lipid-free material is  $1.6 \pm 0.1$  per cent; the values given account for 69 per cent of this; the remainder is probably methionine. The preparation and further analytical data for this material will be reported in another paper.

‡ The cystine added was recovered quantitatively as shown by the agreement of this value, the added cystine sulfur having been deducted, with the value above.

§ This was prepared from horse serum and was purified by crystallizing twice. A solution of the crystals was dialyzed and dried in a vacuum from the frozen state.

*Colorimetric Cystine Determination with Sullivan Reagent*—The alcohol-washed precipitate of the 10 cc. aliquot, after draining, was dissolved in 5 cc. of a solution of 1 per cent hydrochloric acid

<sup>7</sup> A porous porcelain crucible (No. FC-2010) manufactured by the Selas Company, Philadelphia, has been found more convenient than the Gooch crucible. The crucibles furnished at present are very satisfactory. Several now in use have withstood over 100 ignitions in the electric furnace.

in 25 per cent potassium chloride and washed with water into a 15 cc. graduated centrifuge tube, bringing the volume up to about 11 cc. The copper was precipitated in one step, the procedure thus differing from that of Brand *et al.* (6) which was otherwise followed, by adding with stirring 10 drops of pyridine followed by 0.5 cc. of a 10 per cent potassium thiocyanate solution. After dilution to the 15 cc. mark, the dense precipitate was centrifuged out. 5 cc. aliquots of the supernatant fluid were withdrawn for the Sullivan procedure and transferred to 6 × 1 inch test-tubes graduated at 20 cc. This solution was made alkaline with sodium hydroxide (0.5 cc. of 5 N was sufficient). The procedure of Rossouw and Wilken-Jorden (7) was followed for the development of the color. The naphthoquinone reagent was allowed to react 20 seconds before the sulfite was added. After the addition of the final reagent, the sample was diluted to the 20 cc. mark, and then transferred and allowed to stand in the colorimeter tubes 3 or 4 minutes before being read.<sup>8</sup>

The standard used contained 0.80 mg. of cystine. The same reagents (salt, pyridine, thiocyanate) were added to the standard in like quantities as were present in the aliquot of the unknown taken and the color developed as described.

### Results

*Cystine Sulfur by Gravimetric Method*—The data given in Table I show that the values obtained with cystine alone, precipitated as the cysteine mercaptide, were very close to the theoretical. The results obtained with whole spermatozoa or spermatozoa plus cystine agreed within 3.6 per cent. Similar agreement was obtained in several determinations with serum albumin. Considering the numerous steps involved in this determination, these results were considered satisfactory.

*Cystine by Sullivan Method*—The following factors were studied for their effect. (a) Time for action of reagent. The importance of permitting the reaction to continue 20 seconds is discussed by Andrews and Andrews (8). (b) The effect of salts. This error was largely reduced by the use of excess salt (3) which reduced

<sup>8</sup> The Klett-Summerson photoelectric colorimeter was used. The concentration was found proportional to the color developed over a 10-fold range of concentration.

the effect of small total variations in salt content. The excess salt also aided in dissolving the mercaptide precipitate. (c) The effect of copper ion. Small amounts were found to give variable results. The results obtained indicate that the thiocyanate had satisfactorily removed the copper. (d) The effect of pyridine. This was found to be negligible. By consideration of the factors mentioned satisfactory results were obtained, as shown in Table I, the variation being about 4 per cent.

#### DISCUSSION

The Parr bomb served very well to oxidize the mercaptide precipitates for the gravimetric determination of the sulfur. A wet ashing method such as that described by Graff *et al.* (2) would probably be equally satisfactory for oxidizing cysteine.

The use of the Sullivan reaction for the determination of cysteine in the mercaptide precipitates has the advantages of specificity and, compared with gravimetric sulfur determinations, speed. Also smaller samples are required unless a microbalance is available for the gravimetric sulfur determination. The accuracy of the Sullivan method is satisfactory when the precautions mentioned earlier are taken.

Graff *et al.* (2) recommended the determination of nitrogen in the mercaptide precipitates as a measure of the cysteine. However, it has been our experience that the use of the nitrogen content is not satisfactory. The apparent amount of cysteine in precipitates obtained from hydrolysates of spermatozoa was greater when calculated from the nitrogen content of the precipitates than when calculated from their sulfur content. Since purines also give insoluble cuprous compounds (5), the hydrolysis was prolonged from 19 to 30 hours in the hope of completely destroying the purines from the nucleic acid in the spermatozoa,<sup>1</sup> but essentially the same results were obtained. We believe the high nitrogen content of these precipitates was due to the intact purines, although Graff *et al.* (2) stated the hydrolysis described would destroy them. Schultz and Vars (9) have also found extra nitrogen in cuprous oxide precipitates from hydrolysates of liver nucleoprotein and have shown it to be purine nitrogen.

Lucas and Beveridge (4) found precipitation of amino acids other than cysteine to be a cause of high nitrogen values in mer-

captide precipitates. It thus appears that the nitrogen content should be used with caution even in the absence of purines.

The lower cystine sulfur values obtained with spermatozoa with the Sullivan reagent, specific for cysteine, may be significant. Although the difference is close to the limit of error of the method, it appeared consistently. If this is a real difference, possible explanations are non-specific adsorption of other sulfur compounds, precipitation of other sulfhydryl compounds (10), or the occurrence of a limited deamination or decarboxylation of cystine during the hydrolysis.

#### SUMMARY

The application of cuprous oxide for the simultaneous reduction and precipitation as the mercaptide of cystine in hydrolysates of biological material is described. It is pointed out that this procedure is more convenient than the use of zinc for reduction; furthermore, precipitates of zinc phosphate which occur when the material analyzed contains considerable nucleic acid are avoided. The use of the gravimetric sulfur determination and the Sullivan reagent to estimate the cysteine in the mercaptide precipitates is described and compared. The unreliability of the nitrogen determination for this purpose in material containing nucleic acid is pointed out. Some of the precautions to be observed in the use of the Sullivan reagent are discussed.

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## GLUTATHIONE

### I. THE METABOLISM OF GLUTATHIONE STUDIED WITH ISOTOPIC GLYCINE\*

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The administration of the component amino acids of glutathione (GSH) labeled with  $N^{15}$  offers an experimental approach to the study of its metabolism. In this paper we shall describe experiments in which glycine labeled with  $N^{15}$  was administered to rats and rabbits. The uptake of the glycine nitrogen into GSH, protein, and the non-protein nitrogen was measured. Preliminary experiments (1) in which labeled glycine was fed to rats showed that the tripeptide is more active in accepting glycine nitrogen than is the protein of the same organ. A rapid synthesis of GSH in the liver was indicated by the finding of large concentrations of  $N^{15}$  after administration of the labeled amino acid.

For isolation of GSH from organs of rats, tissue was extracted with a solution of metaphosphoric acid; the GSH was precipitated as copper mercaptide. The method of isolating analytically pure GSH from small amounts of tissue was considerably improved later by extracting with trichloroacetic acid and precipitating GSH first as the Cd compound. This procedure and the use of rabbits instead of rats made it possible to obtain enough GSH from rabbit livers to permit the isolation and analysis of the three constituent amino acids.

The results obtained with rabbits were similar to those with rats in respect to the uptake of glycine nitrogen into GSH after

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administration of labeled glycine. The separate isotope analysis of glycine, glutamic acid, and cysteine isolated from liver GSH revealed that 150 minutes after administration of labeled glycine only two-thirds of the total isotopic nitrogen in the peptide was present in its glycine portion, the remaining one-third being in the two other amino acids.

It has been suggested by one of us (2) that GSH may function as an amino acid carrier in processes of detoxication and in the formation of conjugated bile acids, since its component amino acids are those found in combination with compounds other than amino acids in the mammalian body. The isotope technique offered the possibility of testing this idea experimentally. Benzoic acid and labeled glycine were administered simultaneously to rats. The hippuric acid isolated 5 hours later contained an isotope concentration about 3 times higher than that found in the glycine of the liver GSH. This result seems to indicate that the major source of the glycine for the formation of hippuric acid is not the protein or the GSH of the liver.

#### EXPERIMENTAL

*Isolation of GSH As Copper Mercaptide from Small Amounts of Tissue*—In the experiments with rats GSH was isolated by precipitation with copper from extracts of tissue with metaphosphoric acid solution. The analysis of the mercaptide obtained by this method sometimes gave too high ash (calculated for copper) and too low nitrogen values. Moreover the yield of mercaptide corresponded to only 30 to 40 per cent of the average value of about 200 mg. per cent GSH in the liver (3). An improved procedure was based upon Binet and Weller's (4) finding that GSH may be precipitated as the Cd compound from trichloroacetic acid extracts of tissues after neutralization. The method is described in detail for liver but it is in principle the same for all other organs studied (intestinal wall, kidney, brain).

The animals (rabbits, rats) are killed by decapitation. The liver is taken out, freed of the gallbladder (rabbit), and ground in a mortar precooled with dry ice. The ground tissue forms a thin frozen layer on the wall of the mortar. When melting starts, an equal weight of 10 per cent trichloroacetic acid is added.<sup>1</sup> The

<sup>1</sup> The intestine is opened longitudinally, washed with tap water, and dried with filter paper before being ground. It is necessary to repeat the

tissue is thoroughly stirred with the trichloroacetic acid and centrifuged. The extraction is repeated twice with half the original amount of trichloroacetic acid. To the combined extracts a 6 per cent solution of  $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$  is added in an amount equal to one-fourth of the volume of the extract. After the addition of a few drops of brom-cresol green 6 N NaOH is added carefully until the color just turns to a blue-green. Molar sodium bicarbonate is added until the solution is alkaline towards neutral litmus. The precipitate is allowed to settle for 1 hour in the ice box, centrifuged, and washed twice with distilled water with stirring. 2 N  $\text{H}_2\text{SO}_4$  is added to the white cadmium precipitate with stirring until it just dissolves. (The solution remains slightly cloudy.) From 15 to 20 cc. of 0.5 N  $\text{H}_2\text{SO}_4$  for each 50 mg. of GSH expected are then added. The solution is filtered and an aliquot is taken for iodine titration according to Lavine (5).<sup>2</sup> The solution is warmed to 35° and cuprous oxide, prepared from Fehling's solution and glucose and washed neutral, is added dropwise according to the procedure of Hopkins (6). The mercaptide precipitates in shiny crystals which are usually pure white. It is centrifuged and washed three times with 0.5 N sulfuric acid, four times with distilled water, with 50 per cent alcohol until free from sulfuric acid, and then twice with absolute alcohol and dried *in vacuo* over phosphorus pentoxide. If the precipitate is gray, it is washed twice with 0.5 N sulfuric acid, and a cuprous oxide suspension in 0.5 N  $\text{H}_2\text{SO}_4$  is added drop by drop until the mercaptide is dissolved. The solution is filtered rapidly with continued dropwise addition of the oxide suspension to prevent precipitation on the filter. The black residue on the filter is washed with a few cc. of the oxide suspension. The mercaptide begins to precipitate from the filtrate during the filtration. It is completely precipitated by bubbling a stream of air through the solution for about 10 minutes (7).<sup>3</sup> The white mercaptide is washed as described

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freezing and grinding at least once and sometimes twice to obtain a satisfactory preparation of this organ.

<sup>2</sup> This titration gave an approximate estimate of the amount of GSH in the tissue (Table I). In experiments with pure GSH from 80 to 90 per cent recoveries as cadmium compound were obtained.

<sup>3</sup> In later preparations the gray mercaptide was kept suspended in 0.5 N  $\text{H}_2\text{SO}_4$  for several hours (up to 24 hours). From time to time it was centrifuged and the treatment with sulfuric acid was continued until the mercaptide appeared pure white.

above. It remains white if kept over phosphorus pentoxide *in vacuo*. For analysis it is dried at 78° over phosphorus pentoxide *in vacuo* for at least 2 hours. The mercaptide is hygroscopic and when exposed to air rapidly absorbs up to 5 per cent of water (1 mole?).

The analytical values of the mercaptide from rabbit livers are recorded in a foot-note to Table II.<sup>4</sup> The GSH content of the liver as indicated approximately by the iodine titration of the cadmium compound varied widely from animal to animal (see Table II). The yield of GSH isolated corresponded to 55 to 70

TABLE I  
*Administration of Labeled Glycine to Animals*

Ex- peri- ment No.	Animal			Labeled glycine ad- ministered to each animal	N <sup>15</sup> in glycine	Glycine ad- ministered	Experi- mental period
	Species	No.	Weight				
			gm.	mg.	atom per cent excess		hrs.
1	Rat	4	340-390	75	1.18	Subcutaneously	2
2	"	7	300-350	75	1.18	Stomach tube	2
3	Rabbit	1	2090	315	1.18	" "	1
4	"	1	2500	190	1.18	Intravenously	1.5
5	"	1	2100	520	1.18	Stomach tube	2.5
6	"	1	2100	520	1.18	" "	2.5
7	"	1	2460	615	1.18	" "	3.5
8*	"	1	2500	5240	4.92	In diet*	72

\* The rabbit of Experiment 8 had been immunized for other purposes with pneumococcus Type III by Dr. M. Heidelberger. For the present study the liver and intestine were removed after the animal had been killed by exsanguination.

per cent of the titration values in our later preparations. Intestines of rats and rabbits yielded GSH corresponding to 10 to 40 per cent of the titration values. The precipitates obtained from kidney and brain were too small to be investigated further.

*Isolation of Component Amino Acids from GSH*—About 50 to 60 mg. of the mercaptide are hydrolyzed with 5 cc. of constant boiling HCl. Of the three amino acids, glycine is the most difficult one to isolate. To facilitate the isolation of a pure sample of glycine

<sup>4</sup> The Cu and S analyses of the GSCu preparations of the rabbit organs were carried out by Mr. W. Saschek.

TABLE II  
*N<sup>15</sup> Concentration in Nitrogenous Constituents of Liver and Intestine in Animals Which Had Received Labeled Glycine*

Ex- peri- ment No.	Liver						Intestine (small)						Urine	
	GSH			Protein			GSH			Protein			Non-protein nitrogen	
	Titra- tion	Iso- lated* (GSCu)	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	Titra- tion	Iso- lated (GSCu)	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	N <sup>15</sup> con- cen- tra- tion	Amount	
													mg.	atom per cent excess
1	36	21	0.090	0.019	0.040	46†	5	5	0.038	0.017	123	0.014	23	0.030
2	60	20	0.107	0.021	0.032	42	5	5	0.084	0.002	275	0.027	9	0.041
3	68	84	0.013	0.011	0.054	46	34	6	0.008	0.007	61	0.120		
4	90	220	0.018											
5	68	96	0.054	0.004	0.028	79	64	26	0.057	0.007	80	0.082		
6	57	101	0.046	0.016	0.077									
7	58	152	0.065	0.021	0.064									
8	58	159	0.745	0.092	0.534	62							80	0.193

\* Experiment 1, liver GSCu, Cu 17.4 per cent, intestine GSCu, N 11.8 per cent; Experiment 2, liver GSCu, Cu 17.8, N 10.9 per cent; Experiment 3, liver GSCu, Cu 17.12, N 11.27 per cent; Experiment 4, liver GSCu, Cu 17.32, S 8.87 per cent; Experiment 5, liver GSCu, N 11.21 per cent, intestine Cu 17.50, N 11.10 per cent; Experiment 6, liver GSCu, Cu 17.25, S 8.50, N 11.40 per cent; Experiment 8, liver GSCu, Cu 17.37, S 8.57, N 11.13 per cent; calculated for GSCu, Cu 17.26, S 8.66, N 11.35 per cent.

† The melting points of the toluenesulfonylglycine varied from 146.5–148°.

‡ Small and large intestine.

a known amount (Table III) of non-isotopic glycine was added before hydrolysis. The total amount of glycine is thereby increased and its isotope concentration is correspondingly decreased. The addition of normal glycine has the advantage that if glycine is present as an impurity in the other constituent amino acids, it will not affect appreciably the true isotope concentration of the latter.

The solution is concentrated to about 0.5 cc. and saturated with HCl at 0°. The glutamic acid hydrochloride which separates in the ice box during the next 48 hours is filtered on a small sintered

TABLE III  
*N<sup>15</sup> Concentration in Amino Acids of Liver Glutathione*

Ex- peri- ment No.	N <sup>15</sup> concen- tration in GSH	GSCu hydro- lyzed	Gly- cine added	N <sup>15</sup> concentration			
				Found in glycine*	In GSH glycine	In glutamic acid†	In cysteine fraction
	<i>atom per cent excess</i>	<i>mg.</i>	<i>mg.</i>	<i>atom per cent excess</i>	<i>atom per cent excess</i>	<i>atom per cent excess</i>	<i>atom per cent excess</i>
5	0.054	52.3	59.5	0.016 ± 0.003	0.106	0.031 ± 0.003	
6	0.046	56.3	21.8	0.034 ± 0.003	0.099		
7	0.065	85.0	20.0	0.054 ± 0.004	0.116		
8	0.745	83.9	29.8	0.545 ± 0.009	1.50	0.451 ± 0.008	0.190 ± 0.005

\* The toluenesulfonylglycine melted at 148° uncorrected and contained 6.17, 6.07, 6.00, and 5.95 per cent nitrogen respectively (calculated 6.11 per cent).

† The glutamic acid hydrochloride contained 7.77 and 7.73 per cent nitrogen respectively (calculated 7.64 per cent).

glass filter, washed with a few drops of concentrated HCl, and recrystallized twice from HCl saturated at 0°. The cysteine fraction was isolated from the combined glutamic acid filtrate and washings by the procedure of Graff, Maculla, and Graff (8).

The solution remaining after precipitation of the cysteine fraction was freed from copper and zinc with H<sub>2</sub>S. After filtration and washing of the sulfides the filtrate was brought to dryness, the residue was dissolved in 5 cc. of water, and the solution was neutralized to phenolphthalein. 2 moles of *p*-toluene sulfochloride (per mole of glycine expected) were added in ether and the calculated amount of *N* NaOH was added during 6 hours of stirring.

The solution was filtered, neutralized to litmus with HCl, concentrated *in vacuo*, and acidified to Congo red. The toluenesulfonylglycine separated overnight in the ice box and was recrystallized twice from water and from ether with addition of petroleum ether.

*Isolation of Glycine from Protein*—The tissue residue from the isolation of GSH was washed five more times with trichloroacetic acid and hydrolyzed 18 hours with constant boiling HCl. The hydrolysate was freed from HCl *in vacuo*, treated with charcoal, and freed from ammonia by bubbling air through the alkaline ( $\text{Ba}(\text{OH})_2$ ) solution *in vacuo*. After being freed from barium with sulfuric acid the hydrolysate was concentrated to an approximate concentration of 6 per cent nitrogen. Glycine was precipitated from 10 cc. of this solution as the trioxalatochromiate (9). The precipitate was dissolved in 20 cc. of water and the toluenesulfonylglycine was prepared as described above.

*Non-Protein Nitrogen*—The filtrates from the cadmium and copper precipitation of GSH and the trichloroacetic acid washings of the tissue residue were combined for the analysis of non-protein nitrogen.

*Administration of Labeled Glycine to Animals*—Rats and rabbits which had fasted overnight (except in Experiments 4 and 6) were given glycine labeled with  $\text{N}^{15}$  (10) and killed by decapitation at varying intervals thereafter (Table I). Various nitrogenous constituents were isolated as described in the preceding sections and the  $\text{N}^{15}$  concentration was determined (11) (Table II). In Experiments 5 to 8 the GSH of the liver was hydrolyzed and the amino acids were isolated as described above (Table III).

*Administration of Labeled Glycine and Benzoic Acid to Rats*—122 mg. of benzoic acid as sodium benzoate and 150 mg. of labeled glycine (1.98 atom per cent  $\text{N}^{15}$  excess) were administered simultaneously to each of two rats (200 and 210 gm.) by subcutaneous injection. The animals were killed 5 hours later. The combined weight of the livers was 11 gm. The non-protein nitrogen of the liver, obtained by extraction with a solution of metaphosphoric acid, amounted to 18.8 mg. of nitrogen with 0.060 atom per cent  $\text{N}^{15}$  excess. The liver GSH (12 mg. of  $\text{GSCu}$ , N 11.03 per cent) contained 0.151 atom per cent  $\text{N}^{15}$  excess. From 15 cc. of urine 29 mg. of hippuric acid (m.p.  $187^\circ$  uncorrected) were isolated with 1.08 atom per cent  $\text{N}^{15}$  excess.



## DISCUSSION

In the foregoing experiments the metabolic activity of GSH in regard to the utilization of dietary glycine was studied during various periods after administration of labeled glycine. By analysis of the GSH and of the protein of the same organ it was possible to compare their relative activity. The glycine preparations employed contained different isotope concentrations and the isotope values in the isolated samples are thus not directly comparable. In Table IV the values are compared on the assumption

TABLE IV

*Per Cent of Nitrogen of Various Fractions Derived from Nitrogen of Administered Glycine*

The values are calculated for an  $N^{15}$  concentration of 100 atom per cent in the glycine administered.

Experiment No.	Period	Liver				Intestine				Urine
		GSH	Protein	Protein glycine	Non-protein nitrogen	GSH	Protein	Protein glycine	Non-protein nitrogen	
	<i>hrs.</i>									
1	2	7.6		1.6	3.4	3.2		1.4	1.2	2.5
2	2	9.1		1.8	2.7	7.1			2.4	3.5
3	1	1.1		0.9	4.6	0.7	0.2		10.2	
4	1.5	1.5								
5	2.5	4.6	0.3	2.4	7.5	4.8	0.6		7.0	
6	2.5	3.8		1.4	6.5					
7	3.5	5.5		1.8	5.4					
8	72	15.1	1.8	10.8	4.5				3.9	

that all administered glycine samples contained 100 atom per cent  $N^{15}$ . The values thus indicate directly the fractions of each nitrogen sample derived from that of the glycine administered; i.e., in Experiment 1 the GSH of the liver contained 7.6 atom per cent excess. This means that 7.6 per cent of its nitrogen was derived from the administered glycine. In Experiments 5 and 8 the ratio of the "newly incorporated" nitrogen in GSH to that introduced into the protein was 15.2:1 (2.5 hours after administration of the labeled glycine) and 8:1 (in the experiment of 72 hours duration) respectively.

By isolation and analysis of the glycine of GSH and of protein a direct comparison of the uptake of administered glycine was made possible in Experiments 5 to 8 (Tables III and V).

The data show that GSH is much more active in accepting dietary glycine or its nitrogen than is the protein of the same tissue. The introduction of new glycine requires temporary opening and closing of peptide linkages; *i.e.*, partial destruction and resynthesis of GSH. This rapid regeneration of GSH may be demonstrated by an approximate calculation of the time during which half of its glycine is replaced by dietary glycine. Assuming that the isotope content of the glycine available for the GSH synthesis was the same as that fed and that the introduction of labeled glycine approximates a first order reaction, then the half

TABLE V

*Per Cent of Glycine in GSH and Proteins Derived from Administered Glycine*

Experiment No.	Replacement* of glycine in		Ratio of replaced GSH glycine to protein glycine
	GSH	Protein	
5	9.0	2.4	3.8
6	8.5	1.4	6.1
7	9.9	1.8	5.5
8	30.5	10.8	2.8

\* The values in this column are calculated for an  $N^{15}$  concentration of 100 atom per cent in the glycine administered.

time of the reaction (introduction of glycine into GSH) is about 18 hours (Experiments 5 to 7). It is certain that the value of 18 hours is much too high, since beyond doubt the labeled glycine was "diluted" considerably by glycine from other sources such as the non-protein nitrogen and organ proteins. The amounts of GSH obtained from rat liver were too small to permit the isolation of the component amino acids. Assuming that the relative isotope distribution over its 3 nitrogen atoms was the same as in that from rabbits (two-thirds of the total isotope being in the glycine) the reactivity of the GSH must have been much higher. Half of the glycine must have been replaced in less than 8 hours.

The rapid introduction of isotopic nitrogen into the protein of the liver and intestine, in experiments of such short duration

as  $2\frac{1}{2}$  hours in rabbits and 2 hours in rats, shows the participation of dietary glycine nitrogen in protein metabolism immediately after the absorption. This finding is fully in accord with the results obtained after feeding different labeled amino acids during 3 day periods (12). Although the introduction of dietary glycine nitrogen into tissue protein is rapid, the introduction into GSH is much faster. The total amount of GSH is small in comparison with that of the proteins of the same organ. Thus a rapid replacement of glycine in GSH will allow an introduction of only small amounts of the amino acid. The liver proteins take up 5 to 8 times as much glycine in absolute amounts as does the corresponding GSH. The calculation of the absolute amount of glycine that was replaced in the two fractions can only be approximate because of inaccuracies in the determination of total GSH and the possibility that the GSH concentration fluctuated widely during the experiments. Furthermore, only the glycine concentration of the proteins of the whole rat but not of the liver is known from recent analyses of Ratner, Rittenberg, Keston, and Schoenheimer (13).

On the basis of *in vitro* experiments glycine is reported not to be deaminated and not to take part in the process of transamination (14). Recent *in vivo* experiments with rats, however, have shown that glycine yields its nitrogen to (13) and accepts nitrogen from (12) other amino acids. The present study also demonstrates the transfer of glycine nitrogen to other amino acids in very short experimental periods. It was to be expected that most of the isotopic nitrogen in GSH, after feeding marked glycine, was located in the glycine part of GSH; *i.e.*, that most of the new nitrogen was due to replacement of GSH glycine by dietary glycine. In all of the experiments ranging from 2 to 72 hours, 60 to 72 per cent of the total isotope in the GSH was in its glycine (Table VI). Most of the remainder was present in the glutamic acid, demonstrating the fast rate with which glycine nitrogen shifts over to glutamic acid. The isotope concentration of the cysteine fraction (Experiment 8) indicated that the GSH cysteine was much less active than the corresponding glutamic acid. In Experiment 5 the analytical values of glycine and glutamic acid accounted for 84 per cent nitrogen isotope in the GSH. In Experiment 8 the three amino acids were isolated and the recovery of isotopic nitrogen amounted to 96 per cent. Further evidence for the rapidity

of the shift of glycine nitrogen to glutamic acid is the finding that the percentages of the labeled N in the glutamic acid portion of the GSH were almost the same in experiments of such different periods as  $2\frac{1}{2}$  hours and 3 days. The ratio of the isotope concentration in the glycine and the glutamic acid portions of the tripeptide was close to 3:1.

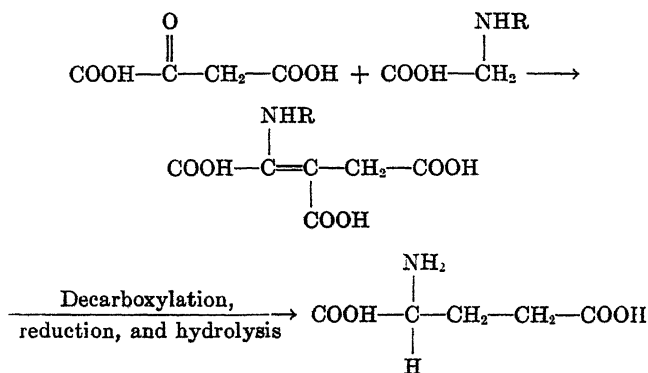
Our experiments give no direct clue to the chemical mechanism of the shift of the glycine nitrogen to glutamic acid. This transfer may have taken place before the incorporation of the constituent amino acids into GSH. If so, glutamic acid must combine rapidly in  $\gamma$  linkage with cysteine in the synthesis of GSH. A second possibility is that the glycine nitrogen may be shifted to the dicarboxylic acid without opening the  $\gamma$  linkage of GSH. A third mechanism might be formation of glutamic acid through a reaction

TABLE VI

*Distribution of Isotopic Nitrogen among Constituent Amino Acids of GSH from Rabbit Liver*

Experiment No.	Duration of experiment	Fraction of isotope in GSH present in		
		Glycine	Glutamic acid	Cysteine
	hrs.	per cent	per cent	per cent
5	2.5	65	19	
6	2.5	72		
7	3.5	60		
8	72	67	20	8.5

between oxalacetic acid and the glycine, the amino group of which is protected by peptide linkage.



An analogous reaction between pyruvic acid and hippuric acid was described by Hoffman (15). GSH may be formed in a similar reaction with oxalacetic acid combined through its  $\beta$ -carboxyl group with cysteinylglycine. Through such a reaction GSH may as an intermediate regulate the needs of the body for glycine. This might explain the negative results obtained with glycine in *in vitro* experiments dealing with deamination and transamination. The last two mechanisms are under investigation.

It may be asked whether the isotopic nitrogen found in the glycine portion of the GSH and of the protein is still attached to the original carbon chain of the dietary glycine. This question is of interest since our experiments show the rapid shift of the glycine nitrogen to other amino acids. The experiments of Schoenheimer, Ratner, and Rittenberg (16) give an approximate estimate of the extent of reamination of glycine. In experiments in which *l*(-)-leucine was fed to rats during a period of 3 days, the isotopic concentration in the glycine of the protein was one-third that in the corresponding glutamic acid. Therefore, if one assumes glutamic acid to hold a central position in the process of nitrogen transfer one may expect that in our experiment only one-tenth of the N in glycine was derived from the glutamic acid.

Cysteine is also involved in the general process of nitrogen transfer, but the uptake of isotopic nitrogen by the cysteine portion of GSH is slower than by glutamic acid.<sup>5</sup> This activity of the amino group may be responsible for the incorporation of stable deuterium from the body fluids into cystine (17).

In the experiment in which labeled glycine and benzoic acid were administered to rats the isotope concentration of the GSH of the liver was only 14 per cent of that of the excreted hippuric acid. Assuming that two-thirds of the labeled nitrogen was in the glycine portion of the tripeptide, as was found in rabbits, the isotope concentration in the hippuric acid would still be 3 times higher than in the GSH glycine of the liver. If the glycine of hippuric acid originated in GSH, the concentration of labeled glycine in the GSH would have to be at least as high as in the hippuric acid during the time when and in the organ where the

<sup>5</sup> Since the cysteine was not isolated as such but the mercaptide precipitate was used for nitrogen determination and isotope analysis, this conclusion is dependent upon the purity of the mercaptide precipitate.

detoxication took place. The comparison of the isotope concentration in the GSH and in the hippuric acid 5 hours after administration offers no support for the assumption that liver GSH participates in the formation of hippuric acid. Still less probable is a direct participation of protein glycine in the formation of hippuric acid because of the much smaller concentration of isotope in the glycine of the protein. The glycine found in hippuric acid might represent a sample of the glycine of the non-protein nitrogen. Wherever the reaction had occurred, the dietary glycine was diluted during the time the synthesis took place by an approximately equal amount of glycine from other sources.

The experiments reported in this paper represent the first endeavor to follow the pathway of one constituent amino acid of a peptide through the animal body. The rapid metabolism of GSH suggests that it may be an intermediate between free amino acids and proteins, transferring<sup>6</sup> amino acids or functioning as a regulating mechanism. Such a function of the tripeptide would be consistent with its rôle in growth and regeneration.

#### SUMMARY

A method for the isolation of analytically pure Cu-glutathione from small amounts of tissue is described. Up to 100 mg. of pure mercaptide were isolated from one rabbit liver. The constituent amino acids of the tripeptide were isolated.

At varying intervals (1 to 72 hours) after the administration of glycine labeled with nitrogen isotope to rats and rabbits the isotope concentration in the GSH and in the protein of liver and intestine was determined. In experiments with rabbits the isotope concentration in the constituent amino acids of GSH and in protein glycine was also measured.

The introduction of glycine N into GSH was much faster than into protein. The absolute amount of new N in protein was greater than in GSH.

About two-thirds of the incorporated isotopic nitrogen found in the GSH was present in the component glycine; the remainder was found in both of the other amino acids. The same relative

<sup>6</sup> If GSH transfers glycine to protein, the time in which half is replaced must be much less than 18 hours, because approximately 5 times as much glycine was incorporated into protein as into GSH in about 3 hours.

distribution was found in experiments of such widely different durations as  $2\frac{1}{2}$  and 72 hours. The concentration of isotope in the glutamic acid in both experiments was about one-third that of the glycine.

Cysteine is involved in the general process of nitrogen transfer among the amino acids.

In one experiment in which benzoic acid and labeled glycine were administered to rats the excreted hippuric acid contained an isotope concentration one-half that of the dietary glycine and 7 times higher than that of the liver GSH.

The findings are discussed in relation to the formation of GSH, its rôle in processes of amino acid transfer, and detoxication.

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# THE IDENTIFICATION AND ESTIMATION OF PENTOSE IN NUCLEIC ACIDS AND NUCLEOPROTEINS

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We have previously reported (1) upon a study of the Dische carbazole reaction (2), and have demonstrated that the method, with certain limitations, can be used for the identification and quantitative estimation of hexose in polysaccharides and glycoproteins. As a continuation of this study we have attempted to apply the method to a study of pentoses and the nucleic acids.

## EXPERIMENTAL

*Pentoses*—A number of pentoses were examined by the carbazole procedure as previously described (1). The Evelyn photoelectric filter photometer (3) was again employed, with filters transmitting maximally at 660, 540, 520, and 420 m $\mu$ . In contrast to the color characteristics of the hexoses obtained after treatment with sulfuric acid and carbazole, the pentoses gave rise to colored solutions which, when tested with our filters, absorbed more strongly at 520 m $\mu$  than at 540 m $\mu$ . In Table I are listed the  $\epsilon_0$  values obtained at 520 and 420 m $\mu$  for a number of pentoses as well as the calculated ratios  $\epsilon_{520}/\epsilon_{420}$ . There was good proportionality between the absorption measured at 520 m $\mu$  and the concentration of pentose. The ratios obtained with arabinose, ribose, and lyxose are very similar, so that they cannot be distinguished qualitatively from one another by this method. Xylose, on the other hand, yielded a very different ratio and can accordingly be readily distinguished from the other pentoses.

*Identification of Pentose in Nucleosides, Nucleotides, and Nucleic Acids*—Adenosine, guanosine, uridine, adenylic acid, and guanylic acid (Table II) yielded  $\epsilon_{520}/\epsilon_{420}$  ratios which agreed closely with the



value of 2.3 to 2.4 given by ribose (Table I). Furthermore, yeast nucleic acid as well as nucleic acid preparations obtained from streptococcus produced colors which indicate that ribose is the sole or main sugar constituent (Table II). In contrast, the ratio obtained with two different samples of thymus nucleic acid was found to be 6.1. It is quite apparent, therefore, that the method can be used to distinguish readily between the two types of nucleic acid.

TABLE I  
*Carbazole Reaction with Pentoses*

Substance	Concentration	$\epsilon_{420}$ *	$\epsilon_{520}$	Ratio $\frac{\epsilon_{520}}{\epsilon_{420}}$
	<i>mg. per cc.</i>			
Arabinose	0.20	0.165	0.375	2.27
"	0.10	0.087	0.189	2.17
"	0.05	0.043	0.097	2.25
Lyxose	0.20	0.145	0.343	2.37
"	0.10	0.073	0.176	2.41
"	0.05	0.040	0.089	2.22
Ribose	0.20	0.155	0.377	2.43
"	0.10	0.081	0.192	2.37
"	0.05	0.043	0.098	2.28
Xylose	0.20	0.092	0.571	6.21
"	0.10	0.046	0.290	6.30
"	0.05	0.024	0.149	6.20
Rhamnose	0.10	0.079	0.108	1.37
Fucose	0.10	0.070	0.083	1.19

\*  $\epsilon_{420}$  refers to  $\epsilon_0$  at 420  $m\mu$ , etc.

*Quantitative Estimation of Pentose*—For the quantitative estimation of pentose, 1 cc. of solution containing 0.05 to 0.10 mg. of pentose is sufficient. The carbazole test was carried out as previously described (1) but the  $\epsilon_0$  values at 520  $m\mu$  (rather than at 540  $m\mu$ ) were employed for quantitative estimations, since the observed maxima were nearer 520  $m\mu$ . For a comparison an appropriate series of pentose standards treated under identical conditions was employed. Satisfactory pentose (ribose) values were obtained with adenylic acid, guanylic acid, adenosine, and guanosine (Table III). Complexes of this type containing purine groups can accordingly be satisfactorily analyzed. On the

other hand, low results were obtained with uridylic acid as well as uridine. This result was not surprising, since it is known that pyrimidine nucleotides and nucleosides are hydrolyzed slowly and incompletely by mineral acids (5). It is clear, therefore, that, although under these conditions the method will serve to characterize the type of pentose present (from the ratio  $\epsilon_{520}/\epsilon_{420}$ ), quan-

TABLE II  
*Carbazole Reactions with Nucleosides, Nucleotides, and Nucleic Acids*

Substance	Concentration	$\epsilon_{420}$	$\epsilon_{520}$	Ratio $\frac{\epsilon_{520}}{\epsilon_{420}}$
	<i>mg. per cc.</i>			
Adenosine . . . . .	0.290	0.124	0.292	2.36
Guanosine. . . . .	0.302	0.119	0.284	2.39
Uridine. . . . .	0.385	0.164	0.357	2.18
Adenylic acid. . . . .	0.289	0.108	0.264	2.42
Guanylic " . . . . .	0.300	0.085	0.206	2.42
Yeast nucleic acid* . . . . .	0.300	0.071	0.146	2.06
" " " † . . . . .	0.406	0.102	0.208	2.06
Streptococcus nucleic acid‡ . . . . .	0.297	0.081	0.184	2.27
" " " § . . . . .	0.381	0.115	0.317	2.76
Thymus nucleic acid  . . . . .	0.092	0.105	0.640	6.09
" " " ¶ . . . . .	0.050	0.061	0.372	6.10

\* Pfanstiehl.

† Kindly supplied by Smith, Kline and French.

‡ Supplied by Dr. M. G. Sevag and Mr. J. Smolens (4). This preparation contained 17.2 per cent reducing sugar (calculated as hexose by the Hagedorn-Jensen method) and gave a negative Feulgen test and a negative diphenylamine test.

§ Supplied by Dr. M. G. Sevag and Mr. J. Smolens (4). This preparation contained 16.5 per cent reducing sugar; negative Feulgen test; weakly positive diphenylamine test.

|| Kindly furnished by Dr. E. G. Kelley.

¶ Kindly furnished by Dr. M. G. Sevag and Mr. J. Smolens.

titative pentose values cannot be obtained with pyrimidine nucleotides because of incomplete hydrolysis of the pyrimidine-pentose linkages. Levene and Jorpes (6) reported that dihydro-uridine- and dihydrocytidinephosphoric acids were readily hydrolyzed by dilute mineral acids, whereas uridine- and cytidine-phosphoric acids were resistant to such treatment. However, conversion of uridine to dihydrouridine by catalytic hydrogenation

(7) proceeds slowly and, since such a process is not easily adapted to microanalytical procedures, attention was turned to the possibility that bromination might be employed. It appears likely, from the work of Levene, that the bonds between pyrimidine and

TABLE III  
*Analyses of Nucleosides, Nucleotides, and Nucleic Acids*

Substance	Ribose			Nitrogen		Phosphorus*	
	Theory	Carbazole method	Modified method	Theory	Found	Theory	Found
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Adenosine . . . . .	56.2	54.8	53.4	26.2	25.6		
Guanosine.. . . .	53.0	49.7	49.6	24.7	20.6		
Uridine .. . . .	61.5	49.3	56.0	11.5	11.1		
Adenylic acid . . . . .	43.2	38.6	40.4	20.2	19.2	8.9	8.7
Guanylic " . . . . .	41.3	43.6	42.2	19.3	18.6	8.5	
Sodium uridyate . . . . .	41.0	13.5	22.0	7.6	7.1	8.4	7.9
Yeast nucleic acid† . . . . .	46.0	25.0	31.5	16.1	13.3	9.5	7.9
" " " ‡ . . . . .	46.0	31.3	35.3	16.1	13.7	9.5	8.3
Streptococcus nucleic acid§. . . . .	46.0	32.4	38.6	16.1	15.3	9.5	8.7
" " "   . . . . .	46.0	46.2¶	50.0¶	16.1	15.5	9.5	8.9

\* We wish to thank Mr. J. Smolens for the phosphorus analyses.

† Pfanstiehl.

‡ Kindly supplied by Smith, Kline and French.

§ Supplied by Dr. M. G. Sevag and Mr. J. Smolens. This preparation contained 17.2 per cent reducing sugar (calculated as hexose by the Hagedorn-Jensen method) and gave a negative Feulgen test and a negative diphenylamine test.

|| Supplied by Dr. M. G. Sevag and Mr. J. Smolens. This preparation contained 16.7 per cent reducing sugar and gave a negative Feulgen test and a weakly positive diphenylamine test.

¶ Since the diphenylamine test was positive, this sample contained a small amount of desoxyribose which gives an intense color with carbazole. The ribose value reported here is therefore too high. This preparation gave a ratio of  $\epsilon_{520}/\epsilon_{420} = 2.76$  (see Table II).

pentose as well as those between pentose and phosphoric acid in pyrimidine nucleotides are weakened considerably by elimination of the 4,5 double bond of the pyrimidine nucleus. Accordingly, uridine was treated in cold, weakly acidic solution with aqueous bromine in the expectation that the 4,5 double bond would

be temporarily eliminated by the formation of 4-hydroxy-5-dibromodihydrouridine just as dibromohydroxyhydrouracil is obtained by bromination of uracil (8). It was found that the products derived by bromination of both uridine and uridylic acid gave significantly higher ribose values than did the original compounds and presumably were more readily hydrolyzed by the sulfuric acid reagent employed (Table III, modified method).

*Modified Carbazole Procedure*—For the quantitative estimation of pentose 3 to 4 mg. of nucleic acid were dissolved in a measured quantity (usually 1 to 2 cc.) of 0.1 N NaOH. The solution was transferred quantitatively to a 10 cc. volumetric flask with distilled water, and enough 0.1 N  $\text{H}_2\text{SO}_4$  was added to neutralize exactly the alkali which had been used. In the case of the nucleosides a slight excess of acid was used in order to make certain that the solution was weakly acidic. The solution was chilled in an ice-water mixture and treated with excess saturated bromine water (usually 2 to 3 drops were sufficient to produce a permanent color). After the solution was allowed to stand in the ice bath for 5 minutes, it was aerated to remove excess bromine. A glass tube drawn out to a capillary is satisfactory for this purpose. After aeration for at least 15 minutes the capillary tube was rinsed with water into the volumetric flask and the solution made up to volume. 1 cc. of this solution was then treated with the sulfuric acid and carbazole reagent as previously described (1). After a 10 minute heating period in a boiling water bath, the solutions were chilled and read in the Evelyn photometer<sup>1</sup> against a blank (containing all of the reagents— $\text{H}_2\text{SO}_4$  reagent, carbazole, and 1 cc. of  $\text{H}_2\text{O}$ ) which had been simultaneously carried through the heating process. The transmission at 520  $\text{m}\mu$  was measured and the  $\epsilon_0$  values ( $-\log$  transmission) at 520  $\text{m}\mu$  were read against a standard curve obtained by similarly determining the  $\epsilon_0$  values at 520  $\text{m}\mu$  for varying concentrations of the appropriate sugar standards. Treatment of the sugar standards with bromine by the method here described does not affect significantly the transmission at 520  $\text{m}\mu$  compared with the transmission of the sugar standards which have not been treated with bromine. Purines as well as pyrimidines in concentrations of 1 mg. per cc. do not interfere with the color development.

<sup>1</sup> Test-tubes with an internal diameter of 19 mm. were employed.

In Table III are listed the results obtained with and without the use of bromine. It will be observed that there was a marked increase in the pentose values when uridine and sodium uridylate were brominated. It was not possible, however, to obtain satisfactory values for uridylic acid. As expected, the values obtained with purine nucleotides and nucleosides remained unchanged regardless of whether or not bromine was used. In the case of the yeast nucleic acids a smaller increase in ribose values was obtained when the modified carbazole method was employed than was the case with uridylic acid (see Table III). The slightly low values found with yeast and streptococcus nucleic acids may be attributed to the incomplete hydrolysis of the pyrimidine-pentose linkages.

*Desoxyribose and Thymus Nucleic Acid*—An authentic sample of desoxyribose<sup>2</sup> (recrystallized once) yielded ratios of  $\epsilon_{520}/\epsilon_{420}$  of 1.4 to 2.0 accompanied by evident signs of decomposition; *i.e.*, upon addition of the carbazole solution to the ice-cold mixture of sulfuric acid reagent and desoxyribose, an intense yellow color was observed. A similar result was obtained with a pure sample of desoxyguanosine<sup>2</sup> (recrystallized four times). This low ratio is in contrast to the value of 6.1 obtained with two differently prepared samples of thymus nucleic acid and similar values given by thymus nucleohistones and placental nucleic acid. Furthermore, these complex substances produced no yellow color when mixed with the cold reagent.<sup>3</sup> In contrast to desoxyribose and desoxyguanosine, there is good agreement between concentrations of thymus nucleic acid and  $\epsilon_0$  values measured at 520 m $\mu$  (Fig. 1). The results obtained with desoxyribose and desoxyguanosine are interpreted as indicative of marked decomposition of the desoxyribose in the strong sulfuric acid employed. The lability of desoxy sugars in strong mineral acids is well known. It is obvious, therefore, that desoxyribose and desoxyguanosine cannot be employed as standards when the carbazole procedure is used.

<sup>2</sup> Dr. P. A. Levene generously supplied us with these substances.

<sup>3</sup> There is good evidence (9) that indicates that the phosphoric acid of thymus nucleic acid is doubly esterified through the alcohol groups at carbon atoms 3 and 5 of the desoxyribose. It is therefore likely that every hydroxyl group of the desoxyribose moiety is combined in the intact nucleic acid molecule.

In Table IV are listed the values obtained with two different samples of thymus nucleic acid as well as several thymus nucleoproteins. The absorption at  $520\text{ m}\mu$  is proportional to the concentration (Fig. 1) and purified thymus nucleic acid may therefore be used as a standard for estimating the nucleic acid content

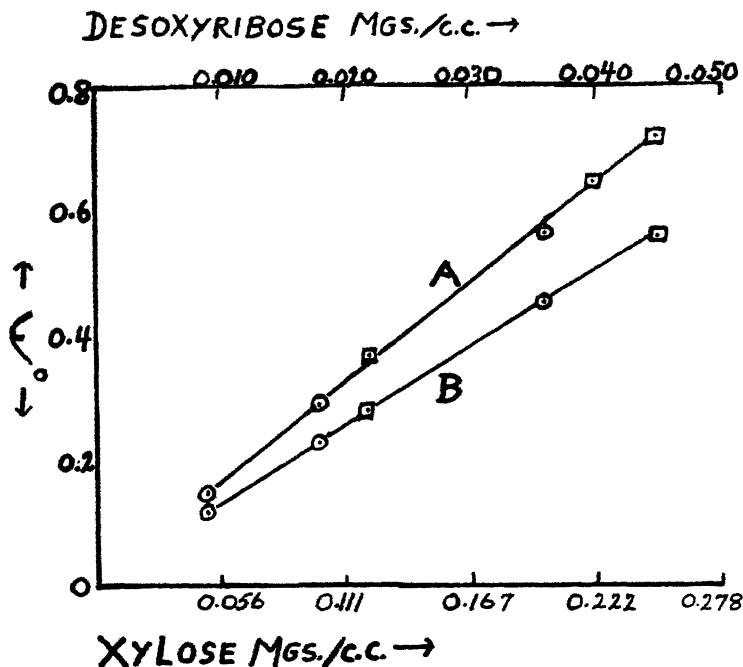


FIG. 1.  $\epsilon_0$  values obtained with xylose and desoxyribose at  $520\text{ m}\mu$  and  $540\text{ m}\mu$  were calculated from the weight of pure thymus nucleic acid employed (thymus nucleic acid  $\times 0.436$  = desoxyribose). The abscissae units on the upper scale are equal to 18 per cent of the units employed on the lower scale. It thus becomes possible to estimate desoxyribose at once by using xylose. The  $\epsilon_0$  values measured at  $520\text{ m}\mu$  (Curve A) and at  $540\text{ m}\mu$  (Curve B) indicate that almost any green filter transmitting in this spectral region may be employed. O = xylose; □ = desoxyribose.

of such nucleoproteins. Since 0.05 mg. of thymus nucleic acid gives a measurable color with carbazole, it is quite likely that as little as 0.025 mg. may be accurately analyzed. It was surprising to find that no increase in color intensity at  $520\text{ m}\mu$  could be observed when the modified carbazole method was employed.

Accordingly, the values listed in Table IV were obtained without preliminary bromination.

The analytical data obtained with thymus nucleic acid (Sample A, Table IV) assure us of the high degree of purity of this specimen. Furthermore, this sample appeared homogeneous when studied in the Tiselius electrophoresis apparatus.<sup>4</sup> Since a number of

TABLE IV  
*Analyses of Thymus Nucleic Acids and Nucleoproteins*

Substance	Ratio $\frac{\epsilon_{520}}{\epsilon_{420}}$	Nucleic acid found	Desoxyribose found*	Nitrogen		Phosphorus	
				Theory	Found	Theory	Found
		per cent	per cent	per cent	per cent	per cent	per cent
Thymus nucleic acid, Sample A† . . . . .	6.10	100‡	43.6‡	16.78	16.76	9.89	9.80
Thymus nucleic acid§. . .	6.09	97	42.4	16.78	15.62	9.89	9.07
Crude placenta nucleic acid   . . . . .	5.32	40	17.4			9.89	3.59
Thymus nucleohistone, rat§. . . . .	5.73	34	14.8		16.70		3.02
Thymus nucleohistone, cat§. . . . .	5.33¶	35	15.3		14.65		2.46

\* The desoxyribose values were obtained by means of the following calculation, (mg. of xylose  $\times$  0.18  $\times$  100)/(weight of sample) = per cent desoxyribose.

† Supplied by Dr. Sevag and Mr. Smolens. This preparation was found to be homogeneous in a Tiselius cataphoresis apparatus (private communication from Dr. Sevag).

‡ These values were *assumed* to be 100 and 43.6 per cent respectively.

§ We thank Dr. E. G. Kelley for these specimens and for the N and P values obtained with these preparations.

|| We thank Dr. S. Graff for this sample of crude nucleic acid prepared from placenta. The purine nitrogen was found to be 3.78 per cent.

¶ A yellow color was produced upon addition of carbazole. It is likely that a contaminant was present and probably produced a low ratio.

different nucleic acid and nucleoprotein preparations made in different laboratories and derived from various sources all yield similar  $\epsilon_{520}/\epsilon_{420}$  ratios (approximately 6) it is a reasonable assumption that the color produced by these specimens may be ascribed to their common desoxyribose moiety. If the amount of desoxy-

<sup>4</sup> See Table IV, † foot-note.

ribose in thymus nucleic acid is assumed to be 43.6 per cent (based on the tetranucleotide unit), then the concentration of desoxyribose may be calculated from the weight of nucleic acid employed (Fig. 1) and the desoxyribose content of various complexes can then be estimated. It should be made clear that we are simply assuming that the above specimen of thymus nucleic acid is pure or very nearly so. This is done only because desoxyribose and desoxyguanosine cannot be used as standards with the carbazole reagent. It is convenient to use xylose as a standard, since it is readily available and produces a color which exhibits characteristics very similar to those of thymus nucleic acid. Our data indicate that the light extinction produced by xylose at 520  $m\mu$  is 18 per cent of that given by equal concentrations of desoxyribose (thymus nucleic acid  $\times 0.436$ ). With xylose as a standard (Fig. 1) desoxyribose may be estimated by means of the conversion factor 0.18 (mg. of estimated xylose  $\times 0.18$  = mg. of desoxyribose). The calculation of the conversion factor obviously rests upon several assumptions and is reported here only because of the need of methods for estimating desoxyribose. Since the desoxyribose content of thymus nucleic acid is undoubtedly a little higher than the assumed 43.6 per cent, the conversion factor of 0.18 is probably slightly low. Obviously, no assumptions need be made if complexes containing desoxyribose are arbitrarily compared against xylose standards and the pentose calculated as xylose.

It must be emphasized that the carbazole method should be employed with great care and can only be used with highly purified materials. The substance tested should obviously be completely free of interfering contaminants, such as polysaccharides, simple sugars, uronic acids, and certain ketones and aldehydes, as well as nitrates and nitrites. With these precautions observed, the two types of nucleic acid can be readily distinguished from each other, and the ribose and desoxyribose contents estimated.

Although the diphenylamine reaction of Dische (2) is highly specific for thymus nucleic acid, larger quantities of material are required for analysis. In view of the apparent destruction of desoxyribose and desoxyguanosine by the carbazole reagent, it was of interest to determine whether desoxyguanosine was destroyed by the diphenylamine reagent. The color produced was found to be very similar to that given by thymus nucleic acid.



Furthermore, there was good proportionality between the concentration and  $\epsilon_0$  values obtained at 660  $m\mu$  (Fig. 2). Desoxyguanosine can accordingly be employed as a standard for the estimation of desoxyribose in thymus nucleic acids and nucleoproteins by the diphenylamine method. When analyzed by this method, thymus nucleic acid Sample A (Table IV) was found to contain 40 per cent desoxyribose. The amount of desoxyribose present therefore accounts for practically all of the carbohydrate believed to be present in thymus nucleic acid.

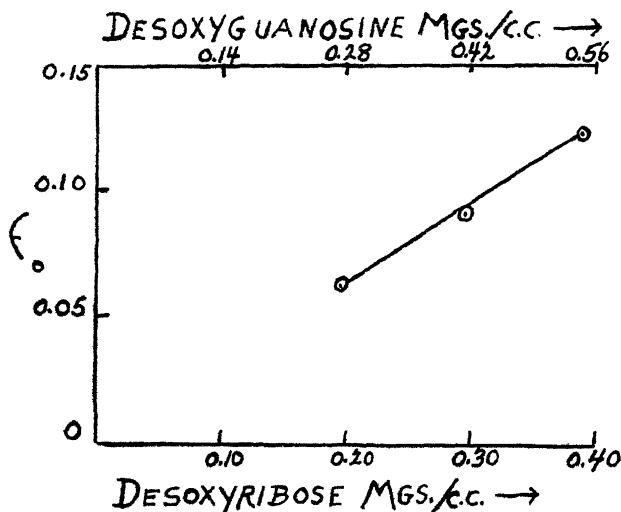


FIG. 2.  $\epsilon_0$  values at 660  $m\mu$  obtained with purified desoxyguanosine. The desoxyribose concentrations were calculated from desoxyguanosine.

#### SUMMARY

Xylose may be distinguished from the other aldopentoses and methyl pentoses by means of the carbazole test.

The procedure may be employed for distinguishing qualitatively between thymus and yeast nucleic acids.

The method can likewise be used for the quantitative estimation of ribose in purine nucleosides and nucleotides.

A modified method involving preliminary bromination improves the determination of pentose in pyrimidine nucleotides and has been utilized for estimating the pentose of yeast nucleic acids.

A procedure has been devised for the estimation of desoxyribose in thymus nucleic acids and nucleoproteins.

The diphenylamine reaction of Dische is more specific and suitable for the estimation of desoxyribose. By means of this test, essentially all of the carbohydrate in purified thymus nucleic acid is accounted for as desoxyribose.

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## THE HEAT OF AN ANTIBODY-ANTIGEN REACTION

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Serological reactions are now generally regarded as essentially chemical in nature. Therefore, if this view is correct, the heat of combination of antibody and antigen should be of a magnitude which appears reasonable in the light of our knowledge of classical thermodynamics.

The first extended study of serological reactions from the physicochemical standpoint was that of Arrhenius (1), who, with Madsen, obtained values for the temperature coefficient of the reaction between tetanolsin and its antibody which enabled them to calculate a heat evolution of 5480 calories per mole of antigen. The first attempt to measure the heat of such reactions directly was that of Bayne-Jones (2) who used a differential microcalorimeter of the type designed by A. V. Hill. A heat evolution of 0.0645 calorie per  $L_f$  unit<sup>1</sup> was observed when diphtheria toxin reacted with antitoxin.

Recent analyses of highly purified toxin indicate that 1  $L_f$  unit contains about 0.00046 mg. of nitrogen. Since the molecular weight of diphtheria toxin is about 70,000 (8), and it contains about 16 per cent of nitrogen, we may calculate that Bayne-Jones' result corresponds to the impossibly large value of  $1.53 \times 10^6$  kilocalories per mole of toxin. Concentrated preparations of toxin, containing as much as 10,000  $L_f$  units per cc., have recently been obtained (7), and were such solutions mixed with the appropriate amount of antitoxin, with comparable volumes, one would

<sup>1</sup> The  $L_f$  unit is the amount of toxin giving most rapid flocculation with 1 standard unit of antitoxin.

predict from Bayne-Jones' result a rise in temperature of over 100°. Smith and Marrack (9) found no detectable rise in temperature (with a thermometer graduated in tenths of a degree) with a toxin containing 500  $L_f$  units per cc. Thus the heat of reaction found by Bayne-Jones seems to be very much in error. Further reason for so thinking lies in the fact that he observed a slow liberation of heat over a period of about 1 hour, contrasting with the extremely rapid rate with which serological reactions are now known to proceed during the first (combination) stage. A possible explanation is that the slow liberation of heat might have been due to the increased heat of stirring of the increasingly viscous reacting mixture. No increase of viscosity would have occurred in the control Dewar flask containing no toxin. The observed temperature difference attributed to the toxin-antitoxin reaction might thus have had a purely mechanical origin. The temperature began to fall very slowly after 72 minutes and this could perhaps have been due to separation of the precipitate attended by a decrease in viscosity. In our own experiments we found a very large change in viscosity.

The most recent attempt to estimate the heat of a serological reaction was that of Follensby and Hooker (5), also with diphtheria toxin and antitoxin. Using a relatively crude method of titration (subject to about 20 per cent error) they found no influence of temperature on the equilibrium of the toxin-antitoxin reaction, and estimated that the heat of reaction was not over 1000 calories per mole of toxin.

The present communication reports a new direct measurement, at 31°, of the heat of a serological reaction, that between the purified hemocyanin of the sea snail, *Busycon canaliculatum*, and its antibody produced by a horse injected with this antigen. The hemocyanins were regularly found to be powerful antigens by Hooker and Boyd (6).

*Preparation of Materials*—The hemocyanin had been purified by repeated precipitation near its isoelectric point. The antigen solution contained 0.9 per cent sodium chloride, added to make it isotonic with the serum. The pH of these reagents, and of samples of normal (non-immune) serum from three different horses, was brought to the same value, as measured by the glass electrode, by addition of the required small amounts of 0.2 N sodium hydrox-

ide or hydrochloric acid. The preliminary experiments were done without addition of preservative to the reactants; in later experiments the addition of merthiolate (Lilly) to a concentration of 0.02 per cent was used to prevent bacterial growth, and resulting alterations of pH.

### *Procedure*

The calorimeter used was previously described by Conn, Kistiakowsky, and Roberts (3). In order to avoid complications due to the heat of precipitation, the reaction was carried out in two stages in the calorimeter. 840 gm. of horse antihemocyanin were put in the outer compartment of the calorimeter, and the inner can was filled with 62 cc. of "weak" hemocyanin. Advantage was taken of the fact that when horse antiprotein serum is mixed with its antigen in such proportions that antibody is in considerable excess no precipitate is formed, although combination takes place between the antibody and antigen. The first quantity of antigen added to the serum slowly produced an opalescent solution, but no precipitate, even on prolonged standing. The calorimeter was brought by electrical heating to a temperature not far from its equilibrium (with stirring) temperature. Observations on the main thermel were made over a suitable interval; whereupon the double plug in the inner can was raised, allowing the antigen to mix with the serum. After heat evolution was complete, the inner can was closed, its contents were pipetted out, and after being rinsed it was filled with 62 cc. of "strong" hemocyanin solution, and the experiment was carried out as before. The concentration of the strong hemocyanin was such as to give a precipitate promptly.

As control experiments, weak and strong hemocyanin solutions were added to normal serum in the manner described above. The concentrations of these solutions are shown in Table I. Runs were made with weak hemocyanin and normal sera from three different horses.

Two series of experiments were made, with portions of the same immune serum and different preparations of hemocyanin. Our results are presented in Table II. The molecular weight of the hemocyanin was assumed to be 6,800,000 (4). The course of the

heat evolution in the first experiment of Series 2, in which weak hemocyanin and immune serum were allowed to react, is shown in Fig. 1. The curve has been corrected for heat of stirring and for heat losses to the outside. Previous experiments have shown that

TABLE I  
*Data Pertaining to Reagents Used in Experiments*

Series No.	Hemocyanin	N per cc.	N in 62 cc.	Hemocyanin in 62 cc. $\times 10^7$	Antibody reacting $\times 10^5$
		<i>mg.</i>	<i>gm.</i>	<i>moles</i>	<i>moles</i>
1	"Weak"	2.03	0.126	1.15	0.98
2	"	2.15	0.133	1.22	1.04
	"Strong"	15.00	0.930	8.51	3.62

The molecular weight of the antigen was assumed to be  $6.8 \times 10^5$ , that of antibody  $1.6 \times 10^5$ . The ratio by weight of antibody to antigen in the compound formed by the addition of "weak" hemocyanin was estimated from analyses of other precipitates to be about 2.0, that in the compound formed when the "strong" hemocyanin was added to be about 1.0.

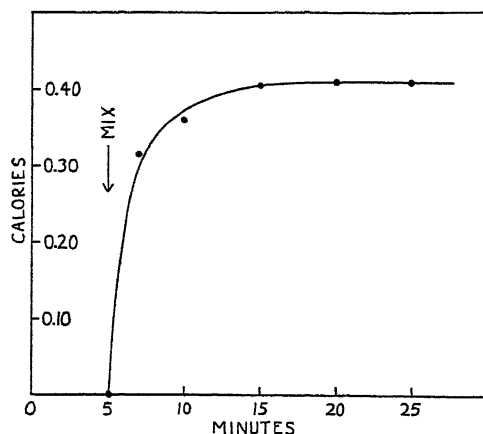


FIG. 1. Evolution of heat when weak hemocyanin and immune serum were allowed to react.

mixing of the contents of the calorimeter is complete after 3 minutes. One may therefore say that the reaction with which we are concerned here is probably a very fast one, since 80 per cent of the heat is evolved within 2 minutes after mixing.

The ratio by weight in which antibody and antigen combine in the region of antibody excess represented by our mixtures of immune serum and weak hemocyanin is not known, since no precipitate is formed. By extrapolating from the results of analyses on precipitates from mixtures in various proportions we

TABLE II  
*Heat Evolved on Mixing Hemocyanin and Normal and Immune Sera*

Series No.		Heat evolved	-ΔH		
			Kilo-calories × 10 <sup>-3</sup> per gm. hemocyanin N	Kilo-calories per mole hemocyanin	Calculated kilo-calories per mole antibody
		<i>calorie</i>			
1	Weak hemocyanin + immune serum, (1)	+0.350			
	Weak hemocyanin + 1st normal serum, (2)	0.000			
	(1) - (2)	+0.350	2.78	3027	35.6
2	Weak hemocyanin + immune serum, (1)	+0.405			
	Weak hemocyanin + 2nd normal serum, (2)	+0.045			
	Weak hemocyanin + 3rd normal serum, (3)	-0.090			
	(1) - ((2) + (3))/2	+0.428	3.58	3900	45.9
	Strong hemocyanin + immune serum, (4)	+0.144			
	Strong hemocyanin + 4th normal serum, (5)	+0.045			
	(4) - (5)	+0.099	0.11	116	2.7*

\* Calculated for antibody reacting with the second addition of hemocyanin.

estimated that in this particular system the maximal ratio would probably be about 2.0. From this estimated value the number of molecules of antibody (assumed to have a molecular weight of 160,000) combining with 1 of antigen, and hence the heat of reaction per mole of antibody, could be calculated. These latter are shown in the last column of Table II.



Two complications were encountered in the first series of experiments. Upon mixing the hemocyanin with immune serum an increase in viscosity occurred, as shown by a very great increase in the heat of stirring, thus making accurate extrapolation to initial time difficult. Furthermore, the solutions were not protected from bacterial growth by preservative, and the experiments with strong hemocyanin in the first series of experiments were worthless.

In the second series of experiments these difficulties were largely remedied. The rate of stirring of the calorimeter was cut in half, thus reducing the heat of stirring to one-eighth its former value. This change resulted in a very much smaller increase in heat of stirring with increased viscosity, making extrapolation to initial time more accurate. The use of merthiolate in the second series of runs appeared to protect the solutions adequately; there was no evidence of bacterial attack.

#### DISCUSSION

The heat of reaction per mole of antigen is large, as one would expect, since it is the resultant of the heats of reaction of a number of molecules of antibody with each antigen molecule. The heat of reaction per mole of antibody is more interesting, since presumably this results from the reaction of a small number of chemical groups. Assuming a reasonable value, say  $-10,000$  calories, for the change in free energy,  $\Delta F^\circ$ , in this reaction, one which goes very nearly to completion but may be reversed experimentally, one may estimate the entropy change,  $\Delta S^\circ$ , from the equation

$$\Delta S^\circ = \frac{\Delta H - \Delta F^\circ}{T} = \frac{-40,000 + 10,000}{304} \cong -100 \text{ entropy units}$$

The entropy decrease in gaseous reactions involving a decrease of 1 unit in mole numbers is usually of the order of 30 entropy units for small molecules, when moles per liter are used as units of concentration. In the antibody-antigen reaction per mole of antibody the mole number decreases by 1 also.

The larger entropy decrease may of course be due entirely to the dissolved state of the molecules or to their large size. On the other hand its magnitude may perhaps be regarded as an indication that several chemical groups are involved in the combination of 1

antibody molecule. It is interesting to note that in the experiment with strong hemocyanin solution considerably less heat, both per mole of antibody and per mole of antigen, is evolved. Two factors may operate to reduce the heat evolution in this experiment. In order to form compounds of lower antibody-antigen ratio, some dissociation of antibody from the original soluble compound must take place, with a resulting heat absorption. Furthermore, it is possible that heat may be absorbed in the process of flocculation. No estimate of the magnitude of this heat change can be made.

We wish to thank the Lederle Laboratories, Inc., for their great kindness in immunizing a horse with hemocyanin and presenting one of us with the resulting antiserum, Dr. Robinson, director of the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health, for the gift of normal horse serum, and the Rockefeller Foundation for its support of the calorimetric work.

#### SUMMARY

With a sensitive calorimeter the heat evolved when an antibody (antihemocyanin from the horse) reacted with its antigen (hemocyanin of *Busycon canaliculatum*) was measured. In the region of antibody excess, where no precipitate was formed, a value of about 3.0 calories per gm. of antigen nitrogen was found (measured at 31°). This corresponds to about 3,300,000 calories per mole of antigen. It is believed that this value is probably accurate to about 20 per cent. By extrapolation from the results of analyses of specific precipitates, it was calculated that the above result corresponds to about 40,000 calories per mole of antibody. Possible factors affecting this value in the same and in different systems are mentioned, and the magnitude of the result is shown to be reasonable from thermodynamic considerations.

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# THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES, WITH RESULTS OBTAINED IN VARIOUS NORMAL TISSUES FROM DIFFERENT SPECIES

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In connection with certain chemical and pathological studies of various tissues, it was desired to obtain quantitative estimates of the contents of collagen and elastin. Since no completely satisfactory methods for measurement of these substances were found in the literature, the methods described here were elaborated. The procedure for measuring collagen has been useful in studying quantitatively the fibrous tissue of normal human myocardium and of the myocardium in cases of coronary arterial disease (1). The methods are also being used in a study of tissue composition; the solids of the extracellular compartment presumably consist of collagen and elastin together with the extracellular salts.

The collagen and elastin contents of 0.2 to 4 gm. samples of muscle have been measured with these methods; satisfactory results are obtained with even smaller samples of tissue containing large amounts of collagen, such as tendon and scar tissue.

The method for measuring collagen consists briefly in extracting from a weighed amount of minced and ground tissue the substances other than collagen and elastin with 0.1 N NaOH at room temperature, washing the residue with water, and neutralizing, defatting, and finally removing the collagen from the elastin and any slight contaminants in the residue, by autoclaving. The difference in the dry weight of the residue before and after autoclaving represents the weight of collagen in the sample analyzed. This method embodies certain parts of the technique used by Schepilewsky (2) in the analysis of the collagen content of large

amounts of tissue. Other investigators have measured collagen by autoclaving the original tissue or the residue following water or salt extractions (3-5), and others have measured collagen after digestion of the other tissue proteins with trypsin (6).

The method of measurement of elastin depends on the insolubility of elastin in boiling 0.1 N NaOH as contrasted with the rapid extraction of the other constituents of the tissue. The weight of the dry residue obtained after the minced and ground tissue is boiled with alkali and washed with water represents the weight of elastin in the tissue analyzed.

### *Procedure*

*Collagen*—Weigh 2 to 4 gm. (4) of finely minced tissue in a 50 cc., round bottom, heavy Pyrex centrifuge tube. Transfer the tissue to a small porcelain mortar and pound and grind until paste-like. Rinse the tissue back into the centrifuge tube with 2 to 4 cc. of water followed by portions of approximately 0.1 N NaOH until the total volume is about 40 cc.<sup>1</sup> Stir vigorously, and let stand overnight. Stir vigorously again, centrifuge, and remove the supernatant fluid by suction. Add 40 cc. of 0.1 N NaOH and stir vigorously. Let stand 2 hours, occasionally stirring, centrifuge, and again remove the supernatant fluid. Add 40 cc. of water and a drop of 0.1 per cent phenol red solution. Adjust the color to faint pink (pH 7) with 0.1 N HCl, stirring vigorously. Sufficient time must be taken to permit diffusion of all the alkali out of the tissue residue before the final adjustment of the pH. Centrifuge, and remove the supernatant fluid. Add 40 cc. of a mixture of 3 parts of 95 per cent alcohol and 1 part of ether and stir well. Allow to stand 10 minutes, stir, centrifuge, and remove the supernatant. Add 40 cc. of ether, stir, allow to settle or centrifuge, and remove the supernatant. Wipe the outside of the tube well with warm water. Dry in an oven at 100° for 2 to 4 hours; i.e., to constant weight. Allow to cool to room temperature and leave in a desiccator until weighed. Weigh the tube

<sup>1</sup> In analyses of liver, spleen, brain, and perhaps some other tissues, it is necessary either to use a volume of original extracting solution 20 times that of the tissue extracted or to replace the first volume of alkali soon after addition by a fresh amount of 0.1 N NaOH; otherwise, the alkaline extract will become so viscous that it cannot be separated properly from the residue.

with contents (B), add 20 cc. of water, plug the tubes with non-absorbent cotton, place in a wire basket, and cover the top with brown paper. Autoclave at 50 pounds pressure for 4 hours, centrifuge, and remove the supernatant fluid. Wipe the outside of the tube. Dry at 100° overnight, cool, and weigh (C).

*Calculation*— $(B - C)/A \times 100 =$  per cent of collagen in the tissue.

*Elastin*<sup>2</sup>—Proceed as for the collagen measurement through the steps of weighing, grinding, and suspending the tissue in 40 cc. of 0.1 N NaOH. Place the tube in a boiling water bath for 10 minutes, centrifuge, and remove the supernatant fluid. Add 40 cc. of 0.1 N NaOH to the residue, place in a boiling water bath again for 10 minutes, centrifuge, and remove the supernatant fluid. Wash the residue with 40 cc. of water, centrifuge, and remove the supernatant. Clean the outside of the tube and dry overnight in an oven at 100°. Cool and weigh. Clean the tube out and dry and weigh the empty tube.

*Calculation*— $(\text{Weight of dry residue})/(\text{weight of sample}) \times 100 =$  per cent of elastin in the tissue.

#### DISCUSSION

*Comments on Procedures*—When small amounts of tissue are handled, the mincing is done preferably in a cold room to prevent evaporation of water. When it is desired to measure the average collagen or elastin content of large amounts of tissue, the entire tissue is finely minced and mixed well. Care is exercised to cut finely and distribute well any particularly fibrous portion of the tissue; a meat grinder is not satisfactory, because it does not cut the fibrous tissue well.

In the procedure for measuring collagen, the extraction of the protein other than collagen and elastin with 0.1 N NaOH at room temperature is much more satisfactory if the tissue is thoroughly ground and pounded to a paste and is well stirred occasionally with

<sup>2</sup> If desired, both collagen and elastin can be measured on the same sample of tissue. Add 40 cc. of 0.1 N NaOH to the residue left in the tube after the collagen has been removed by autoclaving and place the tube in a boiling water bath for 30 minutes. The procedure is carried out from this point according to the directions subsequent to the second boiling with alkali as described in the text.

the alkali. The addition of small amounts of sand to the tissue during grinding has been particularly helpful in accomplishing appropriate mashing of fatty tissue. Approximately 200 mg. of washed sea sand have been used in the analysis of 2 to 4 gm. samples of fatty tissue. Appreciably greater amounts of sand are not recommended, for it was found that the collagen values were approximately 10 per cent higher when 1.5 gm. of sand were added to 2 to 4 gm. samples; apparently small amounts of the finely ground sand were suspended on autoclaving and remained in suspension during the subsequent centrifuging.

When the tissue is minced and ground as described, the same collagen values are obtained when extraction with alkali is accomplished by allowing the mixture of tissue and 0.1 N NaOH to

TABLE I  
*Comparison of Collagen Values Obtained on Tissue Extracted 18 Hours with 0.1 N NaOH with and without Constant Shaking*

Experiment No.	Left cardiac ventricle	Collagen content		
		Without shaking	With constant shaking	Difference
		<i>per cent tissue weight</i>	<i>per cent tissue weight</i>	<i>per cent</i>
1	Dog	0.97	1.01	+4
2	Man	1.08	1.12	+4
3	"	1.09	1.08	-1
4	Dog	1.49	1.41	-5

stand over 1 night as when the mixture is shaken overnight (Table I) or allowed to stand 3 nights (Table II). Studies on heart muscle showed that the tissue, protected so that water loss does not occur, may be kept in the refrigerator for 2 weeks without change in collagen content.

The collagen content has been measured on duplicate samples in most instances. With 2 to 4 gm. samples of muscle, the collagen contents of the two samples usually agree within  $\pm 5$  per cent of their average and rarely differ by as much as  $\pm 10$  per cent. With muscle and other tissues containing approximately similar amounts of collagen, it is preferable to use samples of 2 to 4 gm. when available, although the collagen and elastin contents of samples of 0.2 to 0.5 gm. of tissue may be measured with somewhat less ac-

curacy in the same manner as described above, except that the procedure is carried out in 5 to 15 cc. centrifuge tubes with appropriately smaller amounts of reagents. Obviously, since the amount of collagen in a 0.2 gm. sample of tissue containing 1 per cent collagen is only 2 mg., a more sensitive balance and greater accuracy in weighing are required throughout the procedure when the small samples are utilized. With tissue of very high collagen or elastin content, small samples may be used and the procedures may be carried out either with the large or small tubes.

TABLE II

*Effect of Time of Extraction with 0.1 N NaOH on Collagen Values Obtained on Various Tissues*

Experiment No. and tissue*	Collagen content		
	18 hrs. extraction	72 hrs. extraction	Difference
	<i>per cent tissue weight</i>	<i>per cent tissue weight</i>	<i>per cent</i>
1. Left cardiac ventricle.....	0.92	0.83	-10
2. " " " .....	1.09	1.10	+1
3. " " " .....	0.90	1.01	+12
4. " " " .....	1.02	0.98	-4
5. " " " .....	1.15	1.11	-3
6. Right " auricle ..	3.7	3.6	-8
7. Aorta.....	5.6	5.6	0
8. " .....	8.6	8.0	-8
9. Tendo calcaneus .....	33.2	33.5	+1
10. Skin. ....	34.8	35.2	+1

\* All of these tissues were obtained from human autopsies except the tendo calcaneus which was obtained from autopsy of an elephant.

During the washing with water following the extraction of the tissue with alkali, the watery suspension of the residue is adjusted to pH 7.0. This adjustment of pH is necessary, since some non-collagen protein contaminants would be extracted in addition to the collagen if the medium were too alkaline during the autoclaving. The pH should not be less than 6.0, however, for some collagen would be dissolved and lost during the washing which precedes the autoclaving.

Other investigators (3-5) have employed 2 hours autoclaving at 15 to 20 pounds pressure for removal of the collagen. Studies on



heart muscle and splenic tissue revealed that if autoclaving were continued for 5 to 7 hours at 20 or 25 pounds, the collagen values were 20 to 40 per cent higher than after 2 to 3 hours autoclaving at the same pressure (Table III). In two analyses of aorta, the average value after 7 hours of autoclaving at 20 pounds was 19 per cent higher than after 4 hours autoclaving at the same pressure. When samples were autoclaved for 8 hours at 20 to 25 pounds, the values were not significantly different from those obtained after 6 hours of autoclaving. We routinely employ the procedure

TABLE III

*Effect of Duration and Pressure of Autoclaving on Collagen Values  
Obtained on Various Human Tissues*

The tissues studied included normal and fibrotic myocardium, spleen, liver, and aorta.

No. of samples of tissue	First autoclaving			Second autoclaving*		
	Pressure in autoclave	Time autoclaved	Collagen value	Pressure in autoclave	Time autoclaved	Average increase in collagen values
	lbs.	hrs.	per cent wet weight of tissue	lbs.	hrs.	per cent
4	20	3	3.92	20	4	27
6	25	3	1.12	20	4	35
15	50	3	1.42	20	2	4
8	50	3	1.62	50	3	13
19	50	4	1.92	50	4	8

\* After the dry weight was obtained following the first autoclaving, water was added to the residue and the sample reautoclaved, as indicated in the table.

of autoclaving for 4 hours at 50 pounds pressure; with this procedure, only a slight increase occurs when the samples are autoclaved a second time (Table III). If an autoclave pressure of 25 pounds is to be used, the material should be autoclaved for 6 hours.

Spencer, Morgulis, and Wilder (4) have described a method for measuring the collagen content of muscle in which the dried muscle sample is autoclaved, the gelatin subsequently precipitated with tannic acid, and the nitrogen of the precipitate is measured. In our hands, this method gave higher results by 25 to 200 per cent for the collagen content of three samples of human heart muscle

than were obtained by the method presented above. It was further found that if the residue after removal of the gelatin solution according to the method of Spencer *et al.* was autoclaved again with water, considerably more substance precipitable by tannic acid was obtained. It appears likely, therefore, that the method of Spencer *et al.* gives too high results.

Since different types of collagen apparently exist in tissues, it becomes important to know whether all types are determined by the method presented, and whether or not there is complete specificity. The possible errors which we have considered may be briefly discussed: (a) Although collagen which behaves like tendon will be completely determined, it is possible that there exists in some tissues a type of collagen soluble in cold 0.1 N alkali and therefore not determined by this method. It was at first thought that rat tail tendon might represent such a collagen, since collagen dissolved from this tendon in acetic acid according to the method of Bessey<sup>3</sup> is soluble in 0.1 N NaOH. However, the original rat tail tendon does not dissolve appreciably in 0.1 N NaOH, and it is felt that acetic acid may slightly alter the collagen. It is of interest that weaker alkali (0.01 N) does precipitate the soluble collagen of Bessey, whereas it makes little or no difference in the collagen values obtained from various tissues whether the preliminary extraction is made with 0.1 or 0.01 N NaOH. These findings afford evidence that various types of collagen are determined by the method described here. As pointed out by Mitchell *et al.* (3), strong NaOH (1 N) will extract some of the collagen. (b) It was found that although tendon collagen was hydrolyzed in 2 hours at 15 pounds pressure, some tissues gave higher and more consistent results with longer autoclaving, or autoclaving at higher temperatures. Thus, possibly, some very small amounts of collagen may still resist hydrolysis even after 4 hours of autoclaving at 50 pounds pressure. (c) Since collagen fibers exist in a finely divided state in most tissues, it was thought that they might be more soluble in alkali than tendon strips. However, analysis of tendon preparations scraped into a very fine pulp gave the same collagen values as were obtained with much less finely cut tendon strips. (d) Since a small amount of protein which is neither collagen nor elastin escapes extraction with cold alkali,

<sup>3</sup> Bessey, O., personal communication.

TABLE IV  
*Collagen and Elastin Contents of Various Normal Tissues*

Tissue	Species	Collagen				Elastin			
		No. of tissues analyzed	Per cent wet weight of tissue			Per cent dry weight of tissue, average	Per cent wet weight of tissue		
			Low	High	Average		Low	High	Average
Tendo calcaneus	Elephant	1			20.2	93			0.2
"	Man	1			36.1	86			0.8
Skin	"	2	31.8	34.8	33.3	79			
Sclera	Elephant	1			22.2	76			0.1
Chordae tendineae	Man	2	14.7	19.6	17.2	57			
Aorta	Rat	12	9.0	13.1	11.4	31			
"	Man	5	5.5	8.8	7.1	28	8.0	9.2	8.6
Ligamentum nuchae	Elephant	1			7.6	17	6.9	8.5	7.7
Cardiac auricle	Man	6	3.37	4.04	3.79	15.5			36.2
Right cardiac ventricle	"	8	1.22	2.00	1.58				
"	Dog	6	0.96	1.89	1.38				
"	"	6	0.77	1.32	1.05				
Cardiac ventricular septum	"	5	0.81	1.24	1.02	5.4			
Left cardiac ventricle and septum combined	Man	9	0.59	1.54	1.02	4.8	0.13	0.50	0.31
Spleen	"	21	0.65	2.00	1.00	4.3	0.07	0.37	0.27
Adductor muscle of thigh	Rat	7	0.53	1.49	0.96	3.9	0.08	0.46	0.24
Liver	Man	8	0.65	1.22	0.95				
Ventricular septum	"	8	0.60	1.22	0.91				
Left cardiac ventricle	"	18	0.39	0.86	0.66	3.0	0.26	0.38	0.31
Cardiac ventricles	Rat	8	0.32	0.55	0.46	1.8	0.09	0.16	0.13
Kidney cortex	"	12	0.22	0.66	0.34	1.2	0.04	0.16	0.08
Liver	"	12	0.15	0.48	0.30	1.3	0.18	0.53	0.32
Brain	"	12							1.4

it may be that a little of this will be made soluble on autoclaving and appear in the collagen fraction. However, the hydrolyzed protein solution gave, at most, faint qualitative tests for tyrosine and reduced sulfur, suggesting that little, if any, non-collagenous protein is brought into solution during autoclaving.

### *Collagen and Elastin Contents of Various Tissues*

The collagen and elastin contents of different normal tissues from man and various animals have been measured and are tabulated according to decreasing collagen content in Table IV. Very high collagen values were obtained in tendon, skin, sclera, chordae tendineae, aorta, and cardiac auricle. The collagen content of tendo calcaneus was 93 per cent of the dry weight of the tissue, and the elastin content of this tendon was 0.5 per cent of the dry weight of the tissue. The highest elastin value was observed in ligamentum nuchae with an elastin content of 81 per cent of the dry weight of the tissue and a collagen content of 17 per cent. The average elastin content of several human aortas was 30 per cent and the average collagen content 28 per cent of the dry weight of the tissue. The average collagen content of normal human heart muscle and of the adductor muscles of the thigh of the rat was approximately 5 per cent of the dry weight of the tissues, with an elastin content of 1.5 per cent of the dry weight of the tissues. The brain, on the other hand, showed collagen and elastin contents of only 1.3 per cent and 1.4 per cent respectively of the dry weight of the tissue.

### SUMMARY

1. Simple methods are described for measuring the collagen and elastin contents of 0.2 to 4 gm. samples of tissues.
2. The collagen method here described is considered to be more specific than other available methods.
3. The collagen and elastin contents of various normal tissues in man and in several other species have been presented.

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# FRACTIONATION OF SERUM WITH AMMONIUM SULFATE AND WATER DIALYSIS, STUDIED BY ELECTROPHORESIS

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In recent years, there have been several papers dealing with the inhomogeneity of serum globulin and with the possibility of separating homogeneous components of constant and reproducible properties. Hewitt (7, 8) has reported that there are five different proteins, called euglobulin I and II, pseudoglobulin A, globoglycoid, and the "main pseudoglobulin fraction," the latter not yet exactly defined and still containing impurities of pseudoglobulin A and globoglycoid. Green (3), using isoelectric precipitation, obtained three fractions, called  $P_I$ ,  $P_{II}$ , and  $P_{III}$ , two of which have isoelectric points close to pH 5, and one in the neighborhood of pH 6, in good agreement, as the author herself points out, with the electrophoretic components noted by Tiselius (32). Lately Kendall (10) has succeeded in proving the existence of at least five specific antigens in normal human serum globulin. He has been able to prepare the corresponding antisera and has made progress in isolating the antigens. They have been investigated in this laboratory by sedimentation and electrophoresis. The results will be dealt with in a future paper.<sup>1</sup>

Ultracentrifugal analysis of serum globulin has led to the discovery of three molecular species of serum globulins. The main component has a sedimentation constant of about  $7 \times 10^{-13}$ , one, called the X component, has a less well defined sedimentation constant in the neighborhood of that of serum albumin ( $s = 4.5 \times 10^{-13}$ ), and the third is characterized by its very rapid sedimentation ( $s = 18 - 20 \times 10^{-13}$ ) (Svedberg and Sjögren (28); von

<sup>1</sup> Tiselius, A., Pedersen, K. O., and Svensson, H., to be published.

Mutzenbecher (19); McFarlane (16, 17); Svedberg and Pedersen (27)).

Thus it can be said that each method of investigation has given its own serum globulin fractions, but there still is required a great deal of work, in order to show correlations between all these fractions. Such correlations have already been found between the fractions of Kendall, those of Tiselius, and the ultracentrifugal fractions (Tiselius, Pedersen, and Svensson;<sup>1</sup> Svedberg and Pedersen (27)). It was the purpose of this work to find connections between certain fractions of Tiselius and those obtainable by ammonium sulfate precipitation and by water dialysis. Special interest has been devoted to the fractions described by Hewitt and Green.

### *Methods*

The compositions of the solutions under investigation were followed during fractionation by quantitative electrophoresis experiments in the apparatus of Tiselius (31). Some modifications of the technique originally described have been introduced. The most important of these is the new optical arrangement, developed by Philpot (21) and by the present author (29). The new method is an improvement over the original schlieren method and gives the electrophoresis diagram, also obtainable by the scale method of Lamm (11), as a bright curve on a black background directly on the photographic plate. This facilitates the concentration determinations in a protein mixture. A simple integration of such a diagram gives a quantitative analysis of the solution under investigation, the area under a certain peak being proportional to the concentration of the corresponding component. Of special significance in this connection is the possibility of recording the base-line together with the gradient curve, an advantage that appreciably increases the accuracy of the method. Furthermore, a wedge-formed construction of the inclined slit increases the resolving power for small gradients and makes it possible to record simultaneously peaks of widely different heights. All illustrations in this article have been obtained by the new optical method.

According to a suggestion by Tiselius, a cell twice as high as those originally used was applied in the last experiments of this investigation, in order to get rid of the central horizontal glass

plates, which in the original construction always mask part of the gradient curve. One limb of this cell and the bottom section are filled with the solution to be investigated, the other limb and the rest of the apparatus with buffer. Owing to the hydrostatic pressure of the protein solution column, which tends to cause a sudden movement of the liquid on making the boundaries, this cannot be done without taking great precautions. The difficulty was overcome by using a closed electrode vessel according to Longworth and MacInnes (12), or by adding a calculated amount of buffer in the opposite electrode vessel just before opening the U-tube.

It is not yet known to how great an extent the boundary anomalies (Tiselius (30); Longworth and MacInnes (13)) disturb the concentration-distribution measurements in the electrophoretic analysis of a protein solution. The problem has been discussed by several authors. Some have tried to eliminate the anomalies by using low protein concentrations; others have based their calculations on one side solely, assuming that side to give better results than the other. In the first procedure, it is not advisable, however, to choose too low a protein concentration, while the percentage accuracy in measuring a refractive index gradient necessarily suffers from a decrease in its absolute magnitude, and so the accidental errors in the dilute solutions very easily become larger than the systematic ones in the more concentrated solutions. Without knowledge of the magnitude of the systematic errors due to boundary anomalies nothing can be said about the optimal protein concentration, and so the choice of low concentrations by earlier workers is fully justified.

At first sight the method of basing the calculations on observations on one side only is supported by the fact that the anomalies are more pronounced on the ascending than on the descending side. The method can hardly be justified, however, for although this is true for the most striking anomalies—the illusory boundaries and the erroneous mobilities—it does not hold for other anomalies—salt and protein gradients, superimposed over the normal ones—and only these are of importance for the concentration measurements. Furthermore, the use of both sides for the calculation gives a valuable control, which should not be neglected. There is, however, one case in which the ascending side cannot be used



in the integration; *viz.*, when the "false"  $\delta$ -boundary is not fully separable from some slowly moving real component. In such a case the latter appears much too large, and the integration of the ascending side can give highly erroneous results. Although on the descending side the  $\epsilon$ -boundary causes an error in the same direction, this error is much smaller, and therefore it is advisable to use only this side for the calculation. It must be emphasized, however, that it is preferable to get the anomalous boundaries well separated from all real component boundaries, thus avoiding systematic errors of the kind discussed. Probably this can always be accomplished by choosing a suitable buffer, except when fully uncharged substances are present. In the experiments of this investigation, the "false" boundaries were always easily separable from the slowest real component, which is a guarantee against interference with the concentration measurements.

Tiselius and Kabat (33), in a paper in which the scale method was used for quantitative experiments, found that "the occurrence of a  $\delta$ -boundary does not alter the relative concentration of the other components." This opinion, which was based upon the fact that no appreciable difference was obtained between the analyses derived from the two sides, is equivalent to the assumption that the foreign, superimposed gradients are of magnitudes proportional to the normal ones, which is not unlikely. It is supported by the theory of Henry and Brittain (5), who arrived at the following conclusion: "The advancing column will therefore hold its constituent ions in the same relative proportion as in the original sol, but will in general undergo dilution or concentration." Furthermore, the great number of quantitative analyses made in this work give further evidence in the same direction. In fact, the mean values, obtained from the two sides, as a rule differ less from each other than those from different exposures from the same side; *i.e.*, the eventual systematic errors are smaller than the accidental ones. This has been found to hold for protein concentrations as high as half diluted serum, and possibly it holds for still stronger solutions.

However, marked anomalies must be considered as highly undesirable, for they give rise to a rapid spreading out of the gradients and cause a decreased separation velocity on the descending side, two effects that make trouble in the quantitative analysis. Therefore the boundary anomalies have been depressed to a certain

extent, not by reducing the protein concentration, but by raising the salt concentration to twice the value generally used in serum work. The composition of the buffer was 0.064 M  $\text{Na}_2\text{HPO}_4$  and 0.008 M  $\text{NaH}_2\text{PO}_4$  throughout, which gives a pH of about 7.7 and an ionic strength of 0.20.

In favorable cases (above all when there is a good separation between the peaks) the difference between the percentages from the two sides is generally about 1 per cent, independent of the absolute percentage. This difference is assumed to be a measure of the accuracy.

In regard to the mobility determinations, it is well known that these are seriously affected by the boundary anomalies. Valuable information on this subject has recently been given by Longsworth and MacInnes (13) and by Henry and Brittain (5). In both papers the authors conclude that although more diffuse, the descending boundary yields the correct mobility, while the velocity of the ascending is too high. As these conclusions, however, only refer to systems with one single component, they are not necessarily valid for more than the fastest one in a mixture. In concentrated mixtures, therefore, accurate mobilities cannot be obtained, but they must be derived from special runs in more dilute solutions. In the present investigation, the mobilities have been used for identification of the components only. Special runs for mobility determinations have therefore not been made, and no accurate mobilities can be given.

*Electrophoretic Characteristics of Different Sera*—Sera from four species, horse, cow, swine, and rabbit, have been examined. They showed characteristic differences, not only with regard to the quantitative composition, but also to the number of components and the shape of the peaks.

A diagram of horse serum is shown in Fig. 1. There are not less than six components, which have been called, in the order of decreasing mobilities, albumin,  $\alpha_1$ -,  $\alpha_2$ -,  $\beta_1$ -,  $\beta_2$ -, and  $\gamma$ -globulins. Tiselius (32) in 1937 reported the presence in horse serum of only three globulin peaks, and the same has been found by several other workers. The double nature of the  $\beta$  component in Fig. 1 would perhaps not have been detected by the schlieren method. The two  $\alpha$  components, however, are no doubt due to individual variations in the composition of horse serum.

Cow serum is shown in Fig. 2. Its most characteristic property is the very large  $\gamma$ -peak. In one sample of cow serum, this peak

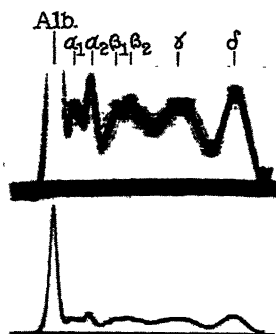


Fig. 1

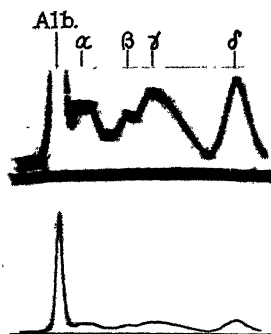


Fig. 2

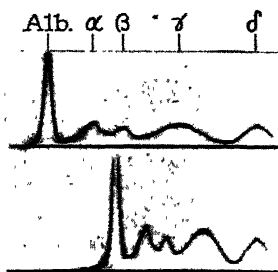


Fig. 3

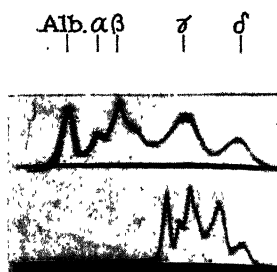


Fig. 4

FIG. 1. Horse serum, diluted 1:2. Exposures taken on the positive side after 222 minutes migration. Angle of inclined slit  $65.0^\circ$  and  $85.0^\circ$  respectively. Potential gradient 5.93 volts per cm.

FIG. 2. Cow serum, diluted 1:2. Exposures taken on the positive side after 204 minutes migration. Angle of inclined slit  $83.0^\circ$  and  $50.0^\circ$  respectively. Potential gradient 6.06 volts per cm.

FIG. 3. Swine serum with reduced albumin content. Exposures taken on the positive side after 57, 184, and 271 minutes migration. Angle of inclined slit  $45.0^\circ$ ,  $70.0^\circ$ , and  $65.0^\circ$  respectively. Potential gradient 6.36 volts per cm.

FIG. 4. Rabbit serum with reduced albumin content. Exposures taken on the positive side after 89 and 200 minutes migration. Angle of inclined slit  $75.0^\circ$  and  $80.0^\circ$  respectively. Potential gradient 6.27 volts per cm.

was distinctly double on both sides.  $\beta$ -Globulin is very small and often difficult to separate from  $\gamma$ -globulin.

Swine serum, as shown in Fig. 3, gave comparatively simple diagrams. The different components separate very beautifully, and the analyses are therefore easy and accurate.

Rabbit serum (Fig. 4) is also a rather simple system, characterized by its very high albumin content.

In the experiments from which Figs. 3 and 4 have been taken the albumin content was reduced to about the same order of magnitude as each globulin component, in order to show better the shape of the globulin peaks (see below).

The opalescence in serum, which is due to fat particles, was found by Tiselius (32), using horse serum, to move with the velocity of  $\beta$ -globulin. It may be of interest, however, to note that in the cow and swine sera here investigated the opalescence migrated with the  $\alpha$  component. A peculiarity that is worthy of note is

TABLE I  
*Per Cent Composition of Horse, Cow, Swine, and Rabbit Sera*

	Albumin	$\alpha$ -Globulin	$\beta$ -Globulin	$\gamma$ -Globulin
Horse . . . . .	43.85	6.75, 9.1	7.4, 13.0	19.9
Cow	53.15	14.45	7.45	24.95
Swine. ....	55.4	13.8	15.8	15.0
Rabbit. . . . .	77.8	4.7	10.3	7.2

the property of the  $\beta$ -boundary to separate into two on the negative side, even if it does not on the positive. One of the  $\beta$ -peaks is always abnormally sharp. The phenomenon was first observed by Longworth, Shedlovsky, and MacInnes (14), who suggested that it may be the result of a reaction taking place owing to the gradual removal from the negative side of some material migrating faster than  $\beta$ -globulin.

The quantitative compositions of the sera are given in Table I. It must be emphasized, however, that Table I is based upon only one or a few animals of each species, and that comparatively large individual variations are possible. The figures for albumin and total globulin agree closely with those given by Hewitt (8); *viz.*, 42, 55, and 76 per cent albumin for horse, ox, and rabbit serum.

*Fractionation with Ammonium Sulfate*—This is undoubtedly the most commonly used method and has found frequent application by numerous workers. It is therefore of great importance to know whether those fractions, already obtained by this method,

are chemically homogeneous, and whether it is possible to prepare new fractions by modifying the procedures hitherto used. Fractions with different solubilities in ammonium sulfate have been examined ultracentrifugally by McFarlane (17) and by Pedersen and Gralén.<sup>2</sup> In both cases it was found that fractions of widely different solubilities gave nearly identical sedimentation diagrams. This result can hardly be fully explained until those very complicated phenomena, met with in studying serum in the ultracentrifuge (interaction between albumin and globulin, sensitivity to small variations in salt concentration, etc.), have been fully penetrated (Pedersen (20); Svedberg and Pedersen (27)).

Different portions of the same serum were mixed, dropwise and under constant stirring, with varying amounts of saturated ammonium sulfate solution. They were allowed to stand overnight in the cold room, and the next day the precipitates were centrifuged down and discarded. The remaining solutions were dialyzed against the buffer to be used in the electrophoresis runs until totally free from sulfate ions. For the dialysis cellophane bags, only slightly larger in volume than the solutions, were used, thus avoiding an undesirable dilution. After complete dialysis the solutions were subjected to electrophoretic analysis by the method already described.

The method of investigating the supernatants instead of the precipitates was suggested by Tiselius, because the risk of irreversible changes is believed to be less in the former than in the latter. Furthermore, the albumin, which is present in the supernatant, can be utilized as a standard in the concentration measurements, the concentrations of the other components being compared with that of this standard. As a matter of fact, the albumin does not precipitate below 55 per cent saturation, and it can be assumed that all concentration changes due to dilution, evaporation, and mechanical adhering to the precipitates (but not coprecipitation) will affect the albumin to the same extent, relatively speaking, as the other components. The presence of such a reference component greatly facilitates the work, making it unnecessary to follow the absolute concentration through all steps in the procedure.

In general, six to seven different ammonium sulfate concentra-

<sup>2</sup> Pedersen, K. O., and Gralén, N., unpublished work.

tions were investigated for each serum; the composition of each sample was determined by electrophoresis, and the concentrations recalculated to give an albumin concentration of 100. The results are given in Table II. An electrophoretic diagram is shown in Fig. 5.

As is clearly demonstrated by Table II, the different sera behave similarly on being salted-out with ammonium sulfate. There is a pronounced parallelism between solubility and mobility. The former increases with the latter. Thus the concentration of  $\gamma$ -globulin decreases very rapidly from the beginning of precipitate formation, and is completely precipitated at relatively low salt concentrations. The faster globulins are characterized by a much wider precipitation range. They start to precipitate at low salt concentrations and are still partially in solution at the highest salt concentrations under investigation. The lack of a range in which the bulk of  $\alpha$ - and  $\beta$ -globulins precipitates indicates a chemical inhomogeneity of a kind that is not reflected in the mobility. This is consistent with recent results of Blix, Tiselius, and Svensson (1) that these fractions are characterized by a great complexity in that they contain a high percentage of phospholipids, cholesterol, and carbohydrates. Possibly they contain different subfractions with different amounts of these non-protein materials, or such subfractions may be formed in the presence of ammonium sulfate, each fraction having its own solubility. As a comparison, it may be mentioned that serum albumin, according to several authors (see for example Sørensen (26) and Hewitt (6)), consists of at least two fractions with different solubilities, although they cannot be separated by electrophoresis (with the possible exception of very prolonged separation; see Blix, Tiselius, and Svensson (1)).

The fact that  $\gamma$ -globulin has a lower solubility than  $\alpha$ - and  $\beta$ -globulins has already been observed by Tiselius (32) whose experiments on this subject are to be considered as preliminaries to this work.

A peculiar behavior is shown by the  $\alpha$ -globulin of rabbit serum in Table II. It first decreases, and then, after 40 per cent saturation, increases. At 60 per cent it reaches a value twice as high as the original one. This result, which is probably not due to experimental error, may be explained by the fact that the albumin, used as reference, contrary to the assumption, begins to

TABLE II

*Composition of Supernatants of Serum, Precipitated with Varying Amounts of Ammonium Sulfate*

Ammonium sulfate saturation	Horse serum				
	Relative concentrations of globulins (albumin = 100)				
	$\alpha_1$ -	$\alpha_2$ -	$\beta_1$ -	$\beta_2$ -	$\gamma$ -
<i>per cent</i>					
0	16	24	19	16	53
25	16	27	16	13	47
30	14	23	13	10	26
40	14	18	11	4	
50	12	11	9	3	
60	13				
	Cow serum				
	$\alpha$ -	$\beta$ -		$\gamma$ -	
0	37	17		52	
35	29	13		14	
40	23	14		7	
45	18	10			
50	21	12			
55	16	11			
	Swine serum				
0	43	21		96	
30	42	15		15	
40	32	13			
50	28	8			
60	26	6			
	Rabbit serum				
0	5.2	16.1		10.2	
25	5.2	11.7		7.8	
30	5.0	9.9		5.7	
35	4.8	9.1			
40	3.2	7.5			
45	3.5	1.3			
50	4.0	4.3			
55	6.3				
60	10.3				

precipitate earlier than at 50 per cent saturation. This point was not, however, investigated further.

At the ammonium sulfate concentrations, 50 to 55 per cent saturation, generally used for the separation of globulin from albumin, considerable quantities of  $\alpha$ - and  $\beta$ -globulins are still in solution. This fraction is apparently lost in the preparation of serum globulin according to the classical method. On purification of the albumin, it goes into the mother liquors.

The discrepancy between the salting-out and the electrophoretic methods for determination of the albumin to globulin ratio in serum has recently been pointed out by Luetscher (15), studying normal and pathological human sera. The differences were especially high in some of the latter cases, and are no doubt due to  $\alpha$ - and  $\beta$ -globulins which are not precipitated by half saturated ammonium sulfate solution, and which often show abnormal increases under pathological conditions.

Hewitt (7) has described a globulin fraction, called globoglycoid, present in serum after half saturation with ammonium sulfate. It therefore appears probable that globoglycoid is identical with the  $\alpha$  and  $\beta$  fractions just mentioned.

According to Hewitt, globoglycoid is prepared in the following way. The filtrate from the globulin precipitation (50 per cent saturation) is acidified to pH 4.7; the precipitate is centrifuged and redissolved in water. This solution is neutralized to pH 7.0, and ammonium sulfate is again added to half saturation. The globoglycoid is then said to appear as a copious precipitate.

The author's first attempts to repeat this procedure constantly failed, no precipitate being formed in the last step. In order to determine the reason for this, electrophoresis diagrams were determined for the "total albumin fraction" (= serum filtrate after 50 per cent saturation), for the precipitate at the acid reaction, and for the filtrate. It was then found that most of the  $\alpha$ - and  $\beta$ -globulins present in "total albumin" were left in the filtrate from the acid precipitation. It was concluded that for the preparation of globoglycoid it is very important to make the precipitation at as high a pH as possible, in order to come into the neighborhood of the isoelectric points (about 5.1) of the  $\alpha$ - and  $\beta$ -globulins.



When, in later preparations, special care was taken to avoid too acid a reaction in precipitating the albumin, globoglycoid was formed, as described by Hewitt. It was reprecipitated twice, dialyzed against buffer, and investigated by electrophoresis.

At the first view, the substance seemed to be rather homogeneous, the bulk of it (about 85 per cent) moving with the velocity of  $\alpha$ -globulin. After a prolonged run, however, the peak began to be unsymmetrical and separated into two. Apparently the  $\beta$  portion present was for a long time masked by the larger  $\alpha$  component. Possibly there is an interaction between the components, resulting in a decreased velocity for the faster, and an increased one for the slower component. The experiments seem to support this view.

Also a small amount of  $\gamma$ -globulin was present in globoglycoid, but no trace of albumin could be detected.

The substance was not further purified, but it seems reasonable that such a purification might lead to a higher degree of homogeneity. At least it can be expected that the  $\gamma$  portion may be removed by isoelectric precipitation (pH 5.1) or by precipitation at the acid side of the isoelectric point.

Recently Rimington and van den Ende (24) reported that globoglycoid after three or four reprecipitations may be obtained in a crystalline form. The crystals were not identical with those of crystalalbumin, but if the crystallization was carried out at pH 4.7, they appeared quite similar to crystalalbumin crystals. The purified material was also found to be free from carbohydrate, contrary to the result of Hewitt who found a very high carbohydrate content and therefore gave the substance the name globoglycoid. Rimington and van den Ende point out that globoglycoid has no globulin properties except the precipitability by half saturated ammonium sulfate at neutral reaction. In all other respects it behaves as an albumin and the authors conclude that crystalalbumin and globoglycoid are identical or closely similar.

This view seems rather unlikely to the present author, since electrophoresis experiments described above show that globoglycoid contains  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins, but no albumin at all. It should be noted that there is a great difference in appearance between the author's globoglycoid and crystalalbumin. The former was obtained as a brown, amorphous mass in the centrifuge tubes,

giving brown, opalescent solutions; the latter was pale yellow in color, and gave quite clear solutions. Hewitt has given a similar description of globoglycoid.

In Rimington and van den Ende's article it is reported that no globoglycoid was formed after the first acid precipitation of albumin. Most probably, therefore, these authors too had used too acid a reaction and lost most of the globoglycoid in the filtrate. The globoglycoid described was obtained after a second acid precipitation, by half saturation at neutral reaction. There is, however, a great risk of precipitating albumin after removing seroglycoid. If the latter has a solubility-increasing effect on globoglycoid, it has certainly a similar effect upon crystalalbumin; so it can be expected that after a couple of recrystallizations the latter is precipitable (partly) by half saturated ammonium sulfate. In fact, the present author, in cases in which no globoglycoid was formed, tried to use 55 per cent saturation instead of 50 per cent. The precipitate obtained in this way, however, had not the same properties as globoglycoid, obtained later, and on electrophoretic investigation it proved to be a rather pure albumin preparation, with only small amounts of  $\alpha$ - and  $\beta$ -globulins. According to the author's experience, globoglycoid cannot be obtained from the crystalalbumin fraction if it is not formed at the first attempt, which will fail if the highest possible pH is not used for the precipitation of crystalalbumin.

To explain the fact that globoglycoid remains in solution at the first half saturation but precipitates at the second, one must assume that seroglycoid has the property of increasing the solubility of other proteins. Such interactions between proteins have been studied by Grönwall (4). He found, for example, that serum albumin has a distinct solubility-increasing effect upon euglobulin.

From the above results the following can be said about the possibilities of preparing electrochemically homogeneous fractions by ammonium sulfate precipitation. Owing to the very broad precipitation range of  $\alpha$ - and  $\beta$ -globulins, the results shown in Table II are, indeed, not very encouraging. It should be borne in mind, however, that no reprecipitations, which would probably have resulted in a better fractionation, have been made in these experiments. Furthermore, there appear to be two procedures which should give fractions of rather good homogeneity, one with

such a low salt concentration that  $\alpha$ - and  $\beta$ -globulins remain in solution, leaving a pure  $\gamma$  preparation in the precipitate, the other with such a high salt concentration that  $\gamma$ -globulin is completely precipitated, leaving in the filtrate a fraction containing a mixture of  $\alpha$ - and  $\beta$ -globulins.

The former method has already been extensively used in serum chemistry, 33 per cent saturation being generally recommended for separation of the globulins into one easily and one sparingly soluble fraction. Of course only the second can be expected to be homogeneous; the first must necessarily contain all three components  $\alpha$ ,  $\beta$ , and  $\gamma$ , according to the results in Table II.

The fraction precipitable by 33 per cent saturation with ammonium sulfate has been prepared by the author from horse and swine sera, the fractions being twice reprecipitated. On electrophoretic investigation they turned out to migrate with a velocity and homogeneity most closely corresponding to those of  $\gamma$ -globulin in serum. This fraction, Globulin 33, is not quite identical with Kendall's " $\alpha$ -globulin" and with "pseudoglobulin A" of Hewitt (Kendall (9); Hewitt (8)), which represent water-soluble fractions of  $\gamma$ -globulin.

To obtain the faster globulins free from  $\gamma$ -globulin, a precipitation limit of about 40 per cent should be suitable according to Table II. This has also been tried for horse, swine, and rabbit sera. The sera were precipitated with 40 per cent ammonium sulfate in the usual manner, centrifuged, and to the supernatants more ammonium sulfate was added to make the solutions 55 per cent saturated. This second precipitate of course holds a certain amount of albumin, but it was not found necessary to remove this by reprecipitation, since a small amount of albumin plays no rôle for the present purpose. The most easily soluble globulin fractions, called Globulin 40/55, were found to contain  $\alpha$ -globulin as the main component, and only traces of  $\gamma$ -globulin. The fact that the latter was at all present seems, at first, to be incompatible with Table II, but is in fact not so. A  $\gamma$ -peak of the order of magnitude of one-tenth of the total globulin would, in these earlier experiments, have been too small to be observed. An electrophoresis diagram of Globulin 40/55 is shown in Fig. 6.

The fractions precipitable by 40 per cent saturation (Globulin 40) were also investigated. These fractions contained only

traces of  $\alpha$ -globulin but large quantities of  $\beta$ - and  $\gamma$ -globulins, the peaks of the latter being impossible to separate completely, even by prolonged electrophoresis.

In Table III the results of the investigation of the different globulin fractions are collected. To sum up, it can be stated that, although there is a great parallelism between mobility and solubility, it is not a simple matter to isolate the electrochemical

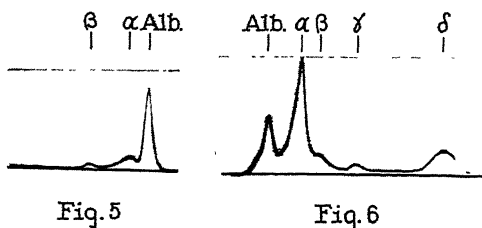


FIG. 5. Supernatant from swine serum, precipitated with 40 per cent saturated ammonium sulfate solution. Exposure taken on the positive side after 158 minutes migration. Angle of inclined slit  $37.5^\circ$ . Potential gradient 6.09 volts per cm.

FIG. 6. Globulin 40/55 from swine serum. Exposure taken on the positive side after 231 minutes migration. Angle of inclined slit  $65.0^\circ$ . Potential gradient 5.90 volts per cm.

TABLE III

*Per Cent Composition of Globulin Fractions Obtained below 33, below 40, and between 40 and 55 Per Cent Saturation of Ammonium Sulfate*

Species	Globulin 33			Globulin 40			Globulin 40/55		
	$\alpha$ -	$\beta$ -	$\gamma$ -	$\alpha$ -	$\beta$ -	$\gamma$ -	$\alpha$ -	$\beta$ -	$\gamma$ -
Horse	0	0	100	8	37	55	49	36	15
Swine	0	0	100	0	17	83	79	14	7
Rabbit				5	31	64			

components by ammonium sulfate fractionation. Furthermore, it must be emphasized that a fraction obtained with the aid of precipitation methods is not necessarily in all respects identical with a certain electrophoretic component, even if it migrates quite homogeneously with the proper velocity. Thus serum albumin prepared according to the classical methods is not identical with electrophoretically isolated albumin. The former is much richer

in the least soluble fraction, the crystalbumin of Hewitt (6), than the latter, and the latter has certainly not the same crystallizability as the former. As another example, it can be mentioned that "Globulin 33" seems to move with a smaller velocity than the bulk of  $\gamma$ -globulin in native serum, a fact that makes complete identity between the two substances improbable.

*Fractionation by Water Dialysis*—The fractions obtained by this method, "pseudoglobulin" and "euglobulin," have been thoroughly studied by numerous workers; the names are often used to denote fractions respectively of high and low solubility in ammonium sulfate. In agreement with Sørensen, Hewitt, and others, pseudoglobulin will be defined here as that globulin fraction which is soluble in distilled water at any pH within the stability region, and euglobulin as that which is precipitable within the same range by removing the salts.

Sørensen (25) showed the impossibility of preparing a euglobulin with constant solubility, even by numerous reprecipitations, and concluded that pseudoglobulin and euglobulin are reversibly dissociable compounds, which can be written as  $E_pP_q$ , where  $p$  and  $q$  can vary widely, but neither of them = 0.

The fractions have also been studied in the ultracentrifuge by Svedberg and Sjögren (28), von Mutzenbecher (18), and McFarlane (17). Regarding pseudoglobulin the results are different, but all investigations hitherto made have shown that euglobulin preparations are polydisperse. Svedberg and Sjögren concluded that the protein was widely denatured and that euglobulin is an artifact, not present in native serum. It should be noticed, however, that electrodialysis has been applied in all the ultracentrifugal studies. It is still questionable whether this method of preparation is to be regarded as a sufficiently gentle one. It has not been much used in later investigations on serum.

As far as the author knows, nobody has considered euglobulin to be a chemical entity, but observations to the contrary have often been made, even by others than those referred to above. Reiner and Reiner (23) found in 1932 that it was possible to prepare euglobulin fractions with different pH values of minimum solubility, and more recently such fractionations have been accomplished by Green (3) and Hewitt (8). The fractions are said to differ from each other in both physical and chemical properties,

and in order to decide whether these differences are reflected in their electrochemical behavior, it appeared worth while to investigate them separately.

According to Hewitt, euglobulin I separates out when serum is dialyzed against water at pH 7, and euglobulin II is obtained from the filtrate if this is acidified to pH 6.

Green's procedure is similar. She dialyzes and acidifies to pH 6.5. The precipitate which is then formed is a mixture of globulins  $P_{II}$  and  $P_{III}$ . The filtrate is acidified to pH 5.0, giving a precipitate of  $P_I$ . To separate  $P_{II}$  from  $P_{III}$ , the first precipitate is dissolved in acid and the solution afterwards adjusted to pH 5.0.  $P_{III}$  then separates out, leaving  $P_{II}$  in solution. The latter is precipitable on addition of more alkali to give pH 6.2.

Apparently Green's globulin  $P_I$  is identical with Hewitt's euglobulin II, even if the pH values recommended by the authors for precipitation of the fractions do not coincide. After having centrifuged down euglobulin I ( $P_{II} + P_{III}$ ), the present author has found the point of maximal precipitation for euglobulin II ( $P_I$ ) to be between 5.0 and 5.3, which agrees better with Green's procedure than with Hewitt's.

Two methods were tried for the investigation of the electrochemical composition of pseudoglobulin and euglobulin and of the fractions of the latter. First, the fractions were prepared as described in the literature and investigated directly by electrophoresis (direct method); second, the composition of serum or serum globulin was determined before and after the precipitation of euglobulin (indirect method). As euglobulin is a rather small part of total serum, a high accuracy is needed in the second method if native serum is used. Such an accuracy is hardly attainable, and experiments of this kind gave no definite results. It is possible, however, to increase the accuracy by depressing the albumin and correspondingly decreasing the globulin concentration. This was done in the following way. Globulin was precipitated from serum in the usual manner with 55 per cent ammonium sulfate, centrifuged, and redissolved in a smaller volume. A suitable amount of the albumin fraction was then added in order to make the albumin peak of the same order of magnitude as the globulin peaks. Such an albumin content is still useful as a reference component, but is not so large as to make a dilution necessary for

avoiding boundary anomalies. Solutions, prepared in this way, gave differences that were much greater than the experimental errors, and definite conclusions could be drawn from the experiments.

Both the direct and indirect methods gave the same results, which indicates that the precipitation and redissolving processes do not change the electrochemical properties.

Euglobulin, obtained by dialysis at neutral reaction (euglobulin I;  $P_{II} + P_{III}$ ) contained mainly  $\gamma$ - and  $\beta$ -globulins, and only traces of  $\alpha$ -globulin, while that precipitated by acidifying to pH 5 turned out to be a rather homogeneous  $\alpha$ -globulin, without a detectable trace of  $\gamma$ -globulin. This is consistent with the method of preparation; it is to be expected that  $\alpha$ -globulin, the most acid component of Tiselius, will have a more acid point of minimum solubility than the others.

Green's procedure for separation of globulins  $P_{II}$  from  $P_{III}$  was also repeated, but owing to lack of time they could not be thoroughly purified, but reprecipitated only once or twice. That was, however, sufficient to show that about 80 per cent of the  $P_{II}$  preparation moved with a velocity in the neighborhood of that of  $\gamma$ -globulin. The fraction  $P_{III}$  was, owing to its extremely low solubility and the very strong opalescence of its solutions, very difficult to investigate. A quantitative analysis could not be made, but it was seen that the bulk of the material moved faster than  $\gamma$ -globulin. Thus Green's assumption that her fractions correspond to those of Tiselius seems to be true, and the correlation should be that  $P_I = \alpha$ -globulin,  $P_{II} = \gamma$ -globulin, and  $P_{III} = \beta$ -globulin.

If we now consider euglobulin as a whole, it is apparent from the foregoing that it contains all three electrophoretic components. The relative amounts have not been determined, but it seems probable that they do not differ significantly from those in native globulin. This is supported by investigations on pseudoglobulin (the filtrate from the second euglobulin precipitate), the composition of which was nearly identical with that of total globulin. *Thus an electrochemical difference between pseudoglobulin and euglobulin cannot be demonstrated; they are both quite as inhomogeneous as total globulin.* In fact, one may speak of at least six serum globulin fractions; viz.,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -pseudoglobulins and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -euglobulins. The reason why, on dialysis against water,

part of the globulin precipitates and part of it remains in solution, is still not clear. Chick's (2) discovery that almost all of the lipid phosphorus of serum globulin is to be found in the euglobulin fraction indicates that non-protein materials in some manner play an important part in the euglobulin formation.

The results related above together with the impossibility of preparing euglobulins with constant properties seem to show that dialysis against water is a rather impractical method for preparing globulin fractions, and that solubility in water is not useful for defining the properties of such fractions.

When this was being written, a paper by Raffel, Pait, and Terry (22) appeared in which these questions are also discussed. On experiments with rabbit serum the authors found that euglobulin contained not only the three globulin components, but also albumin. The latter result is rather surprising, and has not been observed by the present author.

Recently Kendall (9) and Hewitt (8) reported that euglobulin may be obtained by mixing different fractions of pseudoglobulin, and a similar phenomenon is the precipitation of Kendall's  $\alpha$ -globulin (the  $\gamma$ -globulin of Tiselius) at low salt concentrations by the specific polysaccharide of pneumococci. These very interesting reactions have not been studied by the present author and will not be discussed here. It is not unlikely, however, that they will give a valuable contribution to the chemistry of euglobulin.

The question of what significance should be given to the classification of serum globulins in euglobulin and pseudoglobulin forms based upon water solubility is difficult to answer until more is known about the causes of this difference, particularly whether it is of a fundamental nature or only due to irreversible changes in the protein molecules or to more or less loosely bound accessory substances. It remains to be seen whether the pseudoglobulin and euglobulin forms of a certain electrochemically defined fraction are markedly different in other chemical or biological properties. It should be noted that antibody properties in immune sera have been localized to certain electrophoretic fractions (in most cases the  $\gamma$  fraction) more distinctly than has been possible for fractions defined by water solubility. It is hoped that improved methods for preparation in larger amounts and the better purity of fractions studied in this paper will throw further light on this question.



This work was suggested by Professor Arne Tiselius as a continuation of his publications on serum globulin in 1937 (32). The author is greatly indebted to him for facilities, put at his disposal, for valuable advice and instructions, and for good, positive criticism. The author's thanks are also due to Dr. Pedersen for helpful discussions and criticism.

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#### SUMMARY

1. The fractionation of serum globulin with ammonium sulfate at the normal pH of serum was followed by electrophoresis, and it was found that the solubility of the electrophoretic components of Tiselius increases with increasing mobility.

2.  $\alpha$ - and  $\beta$ -globulins were found to have a very broad precipitation range when salted-out from serum. This is related to the great chemical complexity of these components.

3. At 50 per cent saturation of ammonium sulfate  $\alpha$ - and  $\beta$ -globulins are still partially in solution. It has been shown that these remaining globulins are identical with the fraction globoglycoid, described by Hewitt.

4. Globulin fractions, precipitable below 33 per cent and above 40 per cent saturation of ammonium sulfate, were shown to be of a rather good homogeneity. The former, the pseudoglobulin fraction of which is identical with the  $\alpha$ -globulin of Kendall and with the pseudoglobulin A of Hewitt, contained 100 per cent  $\gamma$ -globulin; the fraction precipitated at 40 per cent saturation not earlier described, contained, for swine serum, 79 per cent of  $\alpha$ -globulin.

5. Pseudoglobulin and euglobulin were found to contain all three electrophoretic components in about the same proportions, and no significant difference in the electrochemical behavior could be observed.

6. The fractions euglobulin I and II, described by Hewitt, were found to behave distinctly differently in electrophoresis. The former contained principally  $\beta$ - and  $\gamma$ -globulins; the latter was a rather homogeneous  $\alpha$ -globulin preparation.

7. The fraction  $P_I$  of Green was shown to be identical with euglobulin II of Hewitt and with  $\alpha$ -euglobulin of Tiselius. The

results indicated that Green's fractions P<sub>II</sub> and P<sub>III</sub> correspond to  $\gamma$ - and  $\beta$ -euglobulins respectively.

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# ACTION OF SODIUM SELENITE ON THE OXIDATION OF L-PROLINE

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Wright (1) has made a careful study of the effect of selenium salts in concentrations of 0.0002 to 0.00002 M on the oxygen uptake of tissue slices. He found that after an initial acceleration an inhibition occurred in 60 to 90 minutes. During this inhibitory period added glucose, succinic, lactic, pyruvic, and citric acids were not oxidized. As the oxidation of *p*-phenylenediamine was unaffected, Wright concludes that selenium salts are general dehydrogenase poisons. Potter and Elvehjem (2) showed that M/300 sodium selenite inhibited the oxygen uptake of yeast and the oxidation of added sugars but not of added lactate and pyruvate. The effect of selenite on specific dehydrogenases of mammalian origin has not been thoroughly studied. Collett (3) showed that the succinoxidase of frog muscle was inhibited by 0.2 per cent selenite. Potter and Elvehjem (4) obtained 70 per cent inhibition of the succinoxidase of liver with 0.001 M selenite and Labes and Krebs (5) used 0.02 per cent selenite to inhibit the succinoxidase of pig muscle. Recently Wright (6) showed that urease was inhibited by selenium salts but that liver arginase was not. The concentration of selenium used by these workers, with the exception of Wright, is exceedingly high. It seemed of interest, therefore, to study the effect of selenium as sodium selenite on other specific dehydrogenases to determine their relative sensitivity to the poison.

## EXPERIMENTAL

Since glutathione (1) and possibly other sulfhydryl compounds inhibit the action of selenium, washed tissue was used wherever possible. This preparation was made from rat liver by grinding

the minced liver with sand in a mortar, squeezing through muslin, and washing the resulting suspension three times with 50 cc. of 0.025 M phosphate buffer of pH 7.8 by centrifugation. 1.0 cc. of the resulting pale yellow precipitate was used in each Warburg vessel in a final volume of 2.0 cc. made up with 0.05 M buffer and substrates. This preparation was used for the oxidation of *l*-proline, *d*-proline, succinate, choline, and tyramine. The unwashed liver suspension was used for the oxidation of *l*-tyrosine, alcohol, and hypoxanthine. A brain suspension washed in the same way was used for the oxidation of lactate, pyruvate, and glucose.

An inhibitor such as selenium may act in two ways. It may combine with an active group of the enzyme or it may catalyze an oxidation or some other change in the active group. In the first case the inhibition should occur almost immediately and remain constant with time. In the second case the inhibition may be absent or small at first but should increase with time.

If 0.5 mg. of sodium selenite is added either to the washed or unwashed liver suspension, there is an almost immediate inhibition of the oxidation of all the substrates. If 0.025 mg. ( $8.7 \times 10^{-5}$  M) of selenite is added to the washed preparation, there is an immediate 60 per cent inhibition of the oxidation of *l*-proline but the oxidation of succinate, choline, *d*-proline, and tyramine is unaffected. If this same amount is added to the unwashed preparation, there is an immediate 20 per cent inhibition of the oxidation of *l*-proline and no effect on the oxidation of the four others or on that of *l*-tyrosine, ethyl alcohol, and hypoxanthine. 0.2 mg. of selenite is completely without effect on the oxidation of lactate, pyruvate, and glucose by brain.

If the washed tissue preparation was shaken for varying lengths of time with 0.025 mg. of selenite before the addition of substrate, the succinoxidase became inhibited first, followed in order by the choline oxidase, *d*-proline oxidase, and tyramine oxidase, and after about an hour and a half the inhibitions were all over 70 per cent. The inhibition of *l*-proline which was initially 60 per cent was then only 65 per cent. Controls shaken without selenite were run in all cases. It requires 0.2 mg. of selenite to inhibit the xanthine oxidase 50 per cent and 0.3 mg. to inhibit the *l*-tyrosine oxidase to the same extent. These large amounts are partly necessary be-

cause unwashed tissue had to be used and this may also account for the fact the inhibitions do not increase markedly with the time of incubation. Incubation for 1 hour with selenite has no effect on the brain oxidations.

The differences in the susceptibilities of various enzymes are not correlated with their different concentrations in the tissue preparation. On the basis of the rate of uptake in the first 10 minutes with the washed preparation and with the value of 100 assigned to the succinoxidase, the choline oxidase is 76, *d*-proline oxidase 42, *l*-proline oxidase 31, tyramine oxidase 31. In the unwashed tissue preparation, the *l*-tyrosine, alcohol, and hypoxanthine oxidations are all slower than that of the *l*-proline.

On the basis of these results the various enzymes can be divided into three classes: (a) those relatively insusceptible to selenium, including the glucose, lactate, and pyruvate oxidases of brain and probably *l*-tyrosine, xanthine, and alcohol oxidases of liver; (b) those in which selenium catalyzes the destruction of an active group, including succinoxidase, choline oxidase, *d*-proline oxidase, and tyramine oxidase; and (c) *l*-proline oxidase which is inactivated immediately, suggesting that selenium combines with an active group. The effect of selenium on the *l*-proline oxidase was studied in more detail.

The oxidation of *l*-proline first described in 1932 (7) has not been carefully studied since then. Fig. 1 shows the oxidation of different amounts of *l*-proline by the washed liver preparation. Definite end-points can be obtained corresponding to the uptake of 1 atom of oxygen per molecule. No ammonia is liberated and no phenylhydrazone could be prepared from the end-product. The oxidation is cyanide-sensitive and in the presence of cyanide methylene blue will act as a hydrogen acceptor. *l*-Hydroxyproline is oxidized very slowly by the same preparation (Fig. 1) and no definite end-points could be obtained. The optimum pH for the oxidation lies between 7.8 and 8.1.

Table I shows the effect of washing on the inhibitory effect of selenium on the *l*-proline oxidation. With the unwashed preparation 0.05 mg. caused a 30 per cent inhibition, whereas a washed preparation made from the same liver and containing an equivalent amount of the enzyme is inhibited more than 60 per cent by 0.025 mg. Table I also shows that the enzyme is less stable in the

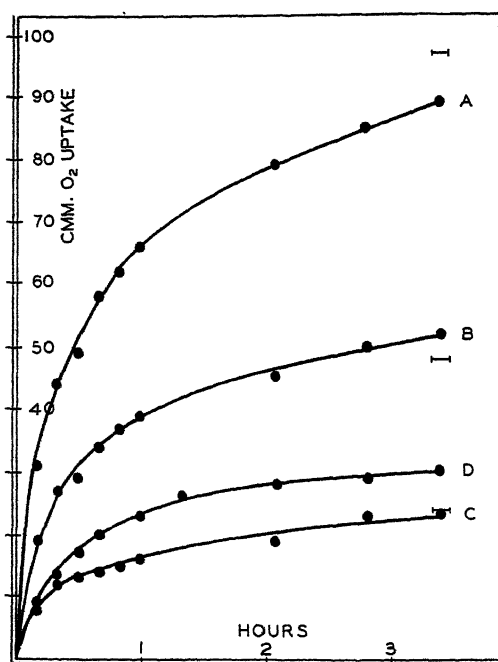


FIG. 1. The oxidation of *l*-proline and *l*-hydroxyproline by washed rat liver preparation. The control oxygen uptake which was small has been subtracted. Curves A, B, C are for 1.0, 0.5, and 0.25 mg. of proline respectively; Curve D, 1.0 mg. of hydroxyproline. pH 7.8, 37°. The horizontal lines represent the theoretical uptakes for the different concentrations.

TABLE I

*Effect of Sodium Selenite on Oxidation of 2.0 Mg. of l-Proline by Washed and Unwashed Rat Liver Suspension*

pH 7.8, 37°. The oxygen uptake of the liver alone has been subtracted from that of the liver plus proline.

Unwashed			Washed			Time	
Proline	Plus 0.05 mg. selenite	Inhibition	Proline	Plus 0.025 mg. selenite	Inhibition		
c.mm.	c.mm.	per cent	c.mm.	c.mm.	per cent	hr.	min.
41	27	34	51	24	63	0	10
68	48	30	73	33	64	0	20
95	68	28	88	41	66	0	30
122	82	33	103	46	68	0	45
183	124	32	121	60	70	1	45

washed than in the unwashed preparation, because the oxidation rate, which was initially about the same, falls off more rapidly in the former. This instability is more marked if the enzyme in the washed preparation is shaken for some time before the addition of *l*-proline. In 1 hour there can be a loss of 50 per cent in activity and this loss is not dependent on the presence of oxygen. Activity cannot be restored by the addition of boiled extract of liver (*Kochsaft*).

Table II shows the effect of adding the selenite to the enzyme 10 minutes before the *l*-proline compared to adding the two simultaneously. After the first 10 minutes the oxidation rate in the two cases is the same, which shows that selenium acts almost im-

TABLE II

*Effect of Adding 0.05 Mg. of Sodium Selenite to Washed Liver Suspension 10 Minutes before 2.0 Mg. of l-Proline Compared with Effect of Adding It Simultaneously with Proline*

pH 7.8, 37°. The oxygen uptake of the respective controls has been subtracted from the figures given.

Proline	Selenite added 10 min. before proline	Selenite added with proline	Time	
<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>hr.</i>	<i>min.</i>
45	9	30	0	10
67	12	34	0	20
83	16	38	0	30
101	20	42	0	45
120	21	43	1	10

mediately. Table III shows the effect of different amounts of tissue suspension on the inhibition by 0.01 mg. of selenite. The rate of oxidation of *l*-proline is no greater with 1.5 cc. of suspension than it is with 1.0 cc.; yet the inhibition of selenite with the former amount is only about half as great as with the latter. The difference in inhibition between 0.5 and 1.0 cc. of suspension is not nearly as marked. Moreover, with the two smaller amounts the inhibition is constant throughout the experiment but with 1.5 cc. it decreases progressively. These facts suggest that even in the washed suspension there are substances that tend to interfere with the action of selenite. All attempts further to purify the *l*-proline oxidase were failures.

Fig. 2 shows the effect of different amounts of selenite on the



TABLE III

*Effect of Concentration of Washed Liver Suspensions on Inhibition by 0.01 Mg. of Sodium Selenite of Oxidation of 2.0 Mg. of l-Proline*

pH 7.8, 37°. The oxygen uptake of the respective controls has been subtracted from the figures given.

0.5 cc. suspension			1.0 cc. suspension			1.5 cc. suspension			Time
Proline	Plus 0.01 mg. selenite	Inhi- bition	Proline	Plus 0.01 mg. selenite	Inhi- bition	Proline	Plus 0.01 mg. selenite	Inhi- bition	
c.mm.	c.mm.	per cent	c.mm.	c.mm.	per cent	c.mm.	c.mm.	per cent	min.
38	8	79	59	19	68	51	32	38	10
55	15	73	82	31	62	75	47	37	20
66	18	73	99	37	62	88	58	36	30
73	20	73	109	42	61	94	64	32	40
83	23	72	118	48	60	102	73	28	50
90	24	73	124	49	61	106	78	26	60

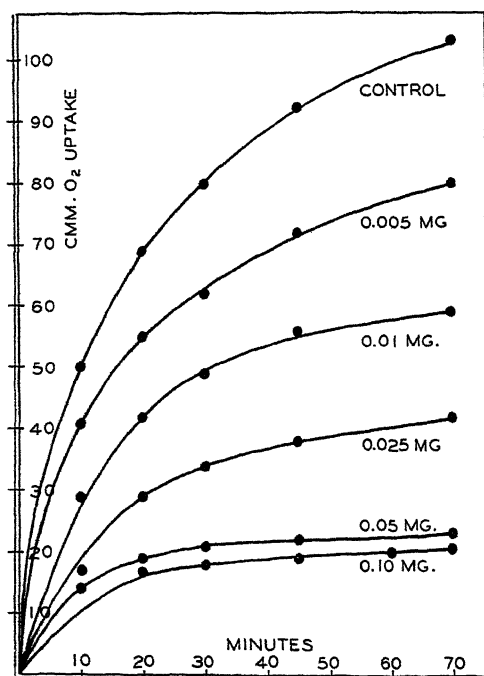


FIG. 2. The effect of different concentrations of selenite ( $1.7 \times 10^{-5}$  M to  $3.5 \times 10^{-4}$  M) on the oxidation of 2.0 mg. of *l*-proline by washed rat liver preparation; pH 7.8, 37°.

oxidation of *l*-proline by a constant amount of washed tissue suspension. Increasing amounts become relatively less effective and the difference between 0.05 and 0.1 mg. is very small. 10 times the latter amount is necessary to obtain complete inhibition. The reason for this is not clear. Varying the proline concentration from 0.5 to 4.0 mg. has no effect on the percentage inhibition by selenite. 1.5 mg. each of arsenite, molybdate, chromate, permanganate, and metavanadate have no effect on the oxidation of *l*-proline under conditions in which 0.01 mg. of selenite will cause a 50 per cent inhibition.

#### DISCUSSION

Wright (1) showed that there was a latent period before selenite inhibited the oxygen uptake of tissue slices alone and in the presence of substrates. This latent period was not caused by a delayed penetration of selenite into the cell, because an immediate acceleration was caused by the salt. In tissue suspensions this acceleration is absent, but unless very large concentrations are used, there is a latent period before selenite inhibits the various enzyme systems tested. The one exception is the *l*-proline oxidase which is inhibited immediately and in concentrations of selenite which would require long latent periods before their effect would be observable on the other enzyme systems.

#### SUMMARY

1. The effect of small concentrations of sodium selenite on the oxidation of various substances by washed and unwashed tissue preparations was studied.
2. There is a latent period before an inhibition is observable on the oxidations of all the substrates except *l*-proline.
3. The oxidation of *l*-proline is inhibited immediately by low concentrations of selenite. 1500 times the concentration of arsenite and certain other salts are without effect.
4. The properties of the *l*-proline oxidase are described.

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## FURTHER STUDIES ON THE RELATIONSHIP OF THE PLANE OF PROTEIN INTAKE TO THE RATE OF NORMAL CALCIFICATION DURING GROWTH

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The experiments here reported continue the work previously published (1) on the rates of calcification during growth in animals receiving diets of different protein content. In the previous work diets of 14.4 and 18.8 per cent protein, respectively, were compared while the present investigation extends the study to a level of protein intake of 25 per cent of the weight of the dry food mixture. The influence of the increased protein intake was studied at two levels of calcium intake; namely, 0.61 to 0.64 and 0.77 to 0.80 per cent of the dry food.

Since the date of our previous paper, Ranganathan and Rau (2) have reported that increasing the calcium content of the ordinary Madras diet results in a more effective utilization of its protein; and Pittman and Kunerth (3), in studies with human adults, have found slightly more favorable calcium balances with medium rather than low intakes of protein. It is doubtful whether any close coordination is to be expected between their investigations and ours, inasmuch as our studies are concerned with calcification during growth.

### *Plan of Present Work*

The plan of this present study was similar to that already described (1), in that young, healthy, albino rats reared to 28 to 29 days of age by mothers on the diets reported in the earlier paper, Diets 171 and 172, containing 18.8 per cent protein and 0.61 and 0.77 per cent calcium, respectively, were grouped into matched lots each consisting of two males and three females. One lot was placed on a diet containing approximately 25 per cent protein and

0.61 per cent calcium, and the other on a diet of similar protein content but containing 0.77 per cent calcium. Food and distilled water were available *ad libitum*. As the sexes were allowed to grow up together, mating occurred as soon as the animals were sufficiently mature.

The offspring of these original matched lots were either used for the formation of other matched lots, so that the effect of the diets might be studied throughout more than one generation, or were reared on the diets of their mothers to various ages, when their bodies were analyzed for calcium content.

The composition of the diets used in the present study is given in Table I, from which it will be seen that the protein content was increased by addition of casein, and the calcium content by

TABLE I  
*Composition of Diets Containing 25 Per Cent Protein and  
0.61 or 0.77 Per Cent Calcium*

Constituents	Diet 673	Diet 675	Constituents	Diet 673	Diet 675
	<i>gm.</i>	<i>gm.</i>		<i>per cent</i>	<i>per cent</i>
Whole wheat... ..	1000.0	1000.0	Ca . . . . .	0.61	0.77
" milk... ..	200.0	200.0	P . . . . .	0.46	0.56
NaCl... ..	20.0	20.0	Protein . . . . .	25.0	25.0
Casein.. . . .	198.0	201.0			
CaCO <sub>3</sub> .....	17.6	18.7			
CaHPO <sub>4</sub> ·2H <sub>2</sub> O... ..		8.5			

addition of calcium carbonate, alone or in combination with calcium phosphate.

#### *Calcium Content of Animals*

As has been previously mentioned, 28 to 29 day-old offspring of the animals on Diets 673 and 675 (both of which contain 25 per cent protein) were reared on the diets of their mothers to 30, 60, 90, and 180 days of age, and their bodies then analyzed for calcium. The technique followed was exactly the same as that previously described (1). The results of these analyses are given in Table II, from which it will be seen that the *percentage* of calcium in the body was slightly higher when the food contained 0.77 than when it contained 0.61 per cent of calcium. This was true for both

sexes and for each of the four ages (30, 60, 90, and 180 days) at which animals were analyzed in this series, covering the period from the end of infancy to adulthood. Lanford's analyses (4, 5) show a similar effect of increased calcium intake (up to 0.8 per cent) with normally nourished animals on diets containing 14.4 per cent of protein; a dietary calcium level of 0.64 per cent sufficed, however, for the attainment of a maximal percentage of calcium at 1 year of age.

TABLE II  
*Average Calcium Content of Rats at Different Ages on Diets Containing  
25 Per Cent Protein and 0.61 or 0.77 Per Cent Calcium*

Sex	Age	Diet No.	Ca in diet	No. of cases	Average net body weight	Total Ca in body	
						gm.	per cent
Males	30	673	0.61	12	53	0.45 ± 0.006*	0.85 ± 0.01*
		675	0.77	12	46	0.46 ± 0.007	0.98 ± 0.007
Females	30	673	0.61	12	50	0.46 ± 0.007	0.88 ± 0.007
		675	0.77	12	44	0.46 ± 0.01	1.02 ± 0.007
Males	60	673	0.61	12	162	1.45 ± 0.02	0.90 ± 0.01
		675	0.77	10	144	1.36 ± 0.03	0.95 ± 0.01
Females	60	673	0.61	12	118	1.28 ± 0.04	1.08 ± 0.01
		675	0.77	16	110	1.22 ± 0.03	1.11 ± 0.01
Males	90	673	0.61	12	235	2.31 ± 0.07	0.98 ± 0.008
		675	0.77	14	218	2.22 ± 0.05	1.02 ± 0.01
Females	90	673	0.61	12	155	1.84 ± 0.05	1.18 ± 0.009
		675	0.77	12	168	2.03 ± 0.04	1.23 ± 0.01
Males	180	673	0.61	13	283	3.02 ± 0.04	1.06 ± 0.006
		675	0.77	11	264	2.85 ± 0.04	1.09 ± 0.01
Females	180	673	0.61	11	187	2.32 ± 0.03	1.24 ± 0.01
		675	0.77	13	186	2.35 ± 0.03	1.27 ± 0.009

\* Probable error.

From the view-point of a consideration of the total *weight* of calcium in the body, it will be seen that there is no marked or constant trend attributable to the difference between 0.61 and 0.77 per cent of calcium in adequate diets at the level of 25 per cent protein in the dry weight of food. For normal animals on this level, as on lower levels of protein intake, the nutritional response appears to be on a plateau from about 0.6 to at least 0.8 per cent of calcium in the food solids.

More detailed examination of the data shows differences which appear to be statistically significant between the females on the two calcium levels at 90 days of age. If the twelve cases at each level are to be regarded as a sufficient number to constitute a

TABLE III

*Average Calcium Content of Rats at Different Ages on Diets Containing from 14.4 to 25.0 Per Cent Protein and 0.61 to 0.64 Per Cent Calcium*

Sex	Age	Diet No.	No. of cases	Ca in diet	Protein in diet	Average net body weight	Total Ca in body	
							gm.	per cent
Males	30	168	30	0.64	14.4	40	0.39 ± 0.003*	0.96 ± 0.004*
		171	5	0.61	18.8	52	0.46 ± 0.01	0.87 ± 0.02
		673	12	0.61	25.0	53	0.45 ± 0.006	0.85 ± 0.01
Females	30	168	17	0.64	14.4	40	0.38 ± 0.006	0.95 ± 0.007
		171	5	0.61	18.8	44	0.42 ± 0.006	0.94 ± 0.02
		673	12	0.61	25.0	50	0.46 ± 0.007	0.88 ± 0.007
Males	60	168	21	0.64	14.4	117	1.13 ± 0.01	0.97 ± 0.006
		171	5	0.61	18.8	148	1.36 ± 0.02	0.92 ± 0.01
		673	12	0.61	25.0	162	1.45 ± 0.02	0.90 ± 0.01
Females	60	168	11	0.64	14.4	106	1.11 ± 0.02	1.05 ± 0.006
		171	5	0.61	18.8	105	1.12 ± 0.03	1.07 ± 0.01
		673	12	0.61	25.0	118	1.28 ± 0.04	1.08 ± 0.01
Males	90	168	31	0.64	14.4	172	1.76 ± 0.03	1.03 ± 0.005
		171	5	0.61	18.8	201	1.96 ± 0.07	0.98 ± 0.03
		673	12	0.61	25.0	235	2.31 ± 0.07	0.98 ± 0.008
Females	90	168	9	0.64	14.4	150	1.75 ± 0.02	1.16 ± 0.008
		171	6	0.61	18.8	122	1.51 ± 0.05	1.23 ± 0.01
		673	12	0.61	25.0	155	1.84 ± 0.05	1.18 ± 0.009
Males	180	168	20	0.64	14.4	282	3.14 ± 0.05	1.11 ± 0.006
		171	5	0.61	18.8	298	3.09 ± 0.11	1.03 ± 0.01
		673	13	0.61	25.0	283	3.02 ± 0.04	1.06 ± 0.006
Females	180	168	8	0.64	14.4	190	2.47 ± 0.03	1.31 ± 0.02
		171	5	0.61	18.8	183	2.26 ± 0.07	1.24 ± 0.01
		673	11	0.61	25.0	187	2.32 ± 0.03	1.24 ± 0.01

\* Probable error.

statistically adequate "sample of the population," it would appear that females on diets fully adequate in all respects and of liberal protein content derive an advantage as to both the amount and percentage of body calcium from an intake of 0.77 over that of 0.61 per cent of calcium in the dry weight of their food. By the

age of 180 days, however, females upon these two diets showed essentially the same body calcium, whether expressed in amount or in percentage.

TABLE IV

*Average Calcium Content of Rats at Different Ages on Diets Containing from 14.4 to 25.0 Per Cent Protein and 0.77 to 0.80 Per Cent Calcium*

Sex	Age	Diet No.	No. of cases	Ca in diet	Protein in diet	Average net body weight	Total Ca in body	
							gm.	per cent
	days			per cent	per cent	gm.	gm.	per cent
Males	30	169	24	0.80	14.4	39	0.40 ± 0.007*	1.02 ± 0.009*
		172	4	0.77	18.8	52	0.48 ± 0.01	0.91 ± 0.02
		675	12	0.77	25.0	46	0.46 ± 0.007	0.98 ± 0.007
Females		169	20	0.80	14.4	38	0.40 ± 0.007	1.05 ± 0.01
		172	5	0.77	18.8	46	0.45 ± 0.01	0.98 ± 0.007
		675	12	0.77	25.0	44	0.46 ± 0.01	1.02 ± 0.007
Males	60	169	16	0.80	14.4	114	1.18 ± 0.02	1.04 ± 0.009
		172	5	0.77	18.8	136	1.35 ± 0.07	0.99 ± 0.03
		675	10	0.77	25.0	144	1.36 ± 0.03	0.95 ± 0.01
Females		169	18	0.80	14.4	96	1.06 ± 0.01	1.11 ± 0.007
		172	6	0.77	18.8	105	1.17 ± 0.03	1.11 ± 0.02
		675	16	0.77	25.0	110	1.22 ± 0.03	1.11 ± 0.01
Males	90	169	30	0.80	14.4	175	1.86 ± 0.04	1.06 ± 0.006
		172	5	0.77	18.8	205	2.09 ± 0.06	1.02 ± 0.01
		675	14	0.77	25.0	218	2.22 ± 0.06	1.02 ± 0.01
Females		169	10	0.80	14.4	154	1.90 ± 0.03	1.24 ± 0.01
		172	5	0.77	18.8	138	1.73 ± 0.04	1.25 ± 0.009
		675	12	0.77	25.0	168	2.03 ± 0.04	1.23 ± 0.01
Males	180	169	20	0.80	14.4	288	3.31 ± 0.04	1.15 ± 0.006
		172	5	0.77	18.8	310	3.33 ± 0.05	1.08 ± 0.005
		675	11	0.77	25.0	264	2.85 ± 0.04	1.09 ± 0.01
Females		169	8	0.80	14.4	189	2.62 ± 0.04	1.38 ± 0.01
		172	6	0.77	18.8	185	2.39 ± 0.03	1.29 ± 0.01
		675	13	0.77	25.0	186	2.35 ± 0.03	1.27 ± 0.009

\* Probable error.

Tables III and IV show, for food calcium levels of 0.61 to 0.64 and of 0.77 to 0.80 per cent, respectively, the comparable data of the present study and of related studies (1, 4) in this laboratory. The data are arranged for convenience at the three levels of protein intake (approximately 14, 18, and 25 per cent of the protein in the food solids). All the animals were of the same hereditary



background and were kept under environmental conditions which were the same except for the experimentally controlled differences in the composition of the food.

From Table III it will be seen that the male animals (all on a diet containing 0.61 to 0.64 per cent calcium) showed, at the ages of 30, 60, and 90 days, higher body weights and lower percentages of body calcium as the result of increasing the protein content of their food from 14.4 to 25.0 per cent. The animals on the diet containing 18.8 per cent protein reached a body weight intermediate between that attained by the animals reared on diets containing 14.4 and 25.0 per cent protein. Such a result was also observed in the case of the percentage of body calcium at 30 and 60 days of age. However, at 90 days the body calcium was similar to that reached by the animals on the other levels of protein intake. On the other hand, the total *amount* of calcium in the body at 30, 60, and 90 days tended to parallel the body weight and probably for this reason to be higher in the animals on the higher protein intakes.

At 180 days of age, however, the differences noted in the preceding paragraph were no longer apparent and the observed deviations among the males on the 14.4, 18.8, and 25.0 per cent protein levels are probably only accidental.

The above facts indicate that in the period of active growth and development (up to 90 days of age) the increased rate of growth induced by higher protein feeding was in excess of the rate of calcification, but that these two phases of development reached a stabilized relationship by the age of 180 days in the rat.

The females showed essentially the same rate of increase in the percentage of body calcium, regardless of whether their food contained 14.4, 18.8, or 25.0 per cent of protein.

From Table IV it appears that increasing the protein content of a diet containing approximately 0.8 per cent of calcium brings about (at least within the limits of 14.4 to 25 per cent protein in the dry food mixture) the same effects in both the male and the female animals as were observed on the diet containing 0.61 to 0.64 per cent of calcium.

#### SUMMARY

1. With rats on a fully adequate diet containing 25 per cent of protein in the dry food, increasing the calcium content of the food

from 0.61 to 0.77 per cent resulted in a slight increase in the percentage of body calcium.

2. When the diet contained 0.61 to 0.64 per cent and when it contained 0.77 to 0.80 per cent of calcium, it was found that increasing the protein content from 14 to 25 per cent of the dry food resulted in an increased rapidity of early growth coincident with a gain in the *amount* of body calcium. The *percentage* of body calcium was not consistently affected in the females; in the males the more rapid gain in body weight induced by the higher protein intake resulted in a retardation of the normal increase in percentage of body calcium, which, however, was essentially the same in the adults regardless of whether they had received 14, 18, or 25 per cent of protein.

3. There was no consistent evidence in either sex that the increased gain in body weight which resulted from the higher of the protein levels here studied had any accelerating effect upon skeletal development as reflected in the *percentage* of body calcium.

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## THE ESTIMATION OF GUANINE AND XANTHINE\*

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Guanine has been determined by a variety of analytical methods. It has been estimated gravimetrically by precipitating the free base with ammonia (1) and as a number of its salts (2). Guanine has been titrated with permanganate (3) and alkaline iodine (4), and it has been estimated by determining guanidine after the oxidation with permanganate (5). Manometric and volumetric methods based on the reaction of the amino group with nitrous acid have been employed (6, 7) and an enzymatic method with a liver guanase preparation has been devised (8). Most of the chemical methods which have been utilized are general in nature, and when a degree of specificity has been attained, as in the method of Robertson (5), the procedures have become unduly complicated. The method to be described here is based on the reaction of guanine (and xanthine) with phenol reagent (9) and is no more specific for guanine than other oxidative procedures. The method has, however, the advantage of requiring much less material than methods already published (0.001 mm is ample for a determination) and in addition is simple and quick and adapted to the determination at one time of a large number of samples. It has been useful for the study of a number of problems in which the composition, qualitatively, of the solutions studied was known or could be determined.

The original observation of the reaction of guanine and xanthine with phenol reagent was made by Funk and Macallum in 1913 (10).

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These authors reported also that adenine does not react with the reagent, and hypoxanthine was stated to give less color than guanine and xanthine (+ instead of ++). The latter observation was an error, for, as will be described, samples of hypoxanthine and adenine when highly purified give no color with the reagent, though ordinary samples usually contain a trace of chromogenic material.

#### EXPERIMENTAL

*Phenol Reagent*—The phenol reagent is most easily prepared by the method given in the last edition of the Folin manual (11). However, when this reagent is used, a precipitate forms during the course of color development. This must be removed before colorimetric comparison. Precipitate formation can be prevented by the addition of lithium sulfate (150 gm. per liter of reagent), as was done in the preparation of Folin and Ciocalteu (12).

*Sodium Carbonate*—A solution of  $\text{Na}_2\text{CO}_3$  saturated at room temperature.

*Standard*—29.4 mg. of guanine hydrochloride ( $\text{C}_5\text{H}_5\text{N}_5\text{O} \cdot \text{HCl} \cdot \text{H}_2\text{O}$ ) are dissolved in 100 cc. of 0.1 N HCl. 1 cc. of this solution contains 0.1 mg. of N (0.001428 mm of guanine).

#### Procedure

A quantity of unknown solution to contain between 0.0007 and 0.0028 mm of guanine or xanthine is measured into a test-tube calibrated to contain 25.0 cc. A 1 cc. portion of the standard is measured into a similar tube. 1.5 cc. of the phenol reagent and 8 cc. of sodium carbonate solution respectively are added to each tube. The solutions are diluted to the mark, mixed, and placed in a beaker of water at a temperature of 40–50°. The whole then is allowed to stand (while it cools gradually) for 20 minutes. At the end of this time the solutions are compared in a colorimeter with the standard guanine solution.

#### Results

The results of a number of experiments with standard solutions of adenine, guanine, xanthine, and hypoxanthine are given in Table I. From these data it can be seen that with either guanine (Experiments 1 to 4) or xanthine (Experiments 5 to 7) the color

developed is proportional to the amount of purine present within the limits of error of the colorimetric readings. Furthermore, the color is not altered by the presence of adenine or hypoxanthine (Experiments 8 to 17). The latter substances, therefore, do not affect the color development, and moreover may be assumed to have no chromogenic power. The color developed by xanthine

TABLE I

*Color with Phenol Reagent Developed by Various Purines*

Each solution was compared in a Dubosq colorimeter against a solution containing 1.428 micromoles of guanine (0.1 mg. of guanine N) as a standard.

Experiment No.	Composition of solution					Chromogenic substance found	
	Guanine	Xanthine	Hypoxanthine	Adenine	Oxyadenine		
	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>per cent</i>
1	1.43	0	0	0		1.42-1.45	99-103
2	0.71	0	0	0		0.71	99.6
3	1.07	0	0	0		1.08	100.7
4	2.86	0	0	0		2.89	101.0
5	0	1.43	0	0		1.43	100.0
6	0	2.14	0	0		2.13	99.3
7	0	2.86	0	0		2.86	100.0
8	1.43	0	8.9	0		1.44	100.5
9	1.43	0	17.9	0		1.43	100.0
10	1.43	0	35.7	0		1.42	99.5
11	1.43	0	71.4	0		1.44	100.5
12	1.43	0	0	14.3		1.42	99.5
13	1.43	0	0	28.6		1.42	99.0
14	1.43	0	0	42.8		1.43	100.0
15	1.43	0	0	71.4		1.44	101.0
16	0	1.43	71.4	0		1.44	100.5
17	0	1.43	0	57.1		1.44	100.5
18	0.714	0.714	0	0		1.43	100.0
19					1.43	1.42	99.0

and guanine is strictly additive (Experiment 18). Oxyadenine (2-oxy-6-aminopurine) reacts with the phenol reagent in the same manner as do xanthine and guanine (Experiment 19).

Not all samples of adenine and hypoxanthine are free from chromogenic substances. In fact most preparations contain color-producing substances equivalent to 0.5 to 1 per cent of guanine

(Table II). The pure preparations used in the experiments for which the data are given in Table I were prepared as follows:

*Adenine*—Adenine picrate was recrystallized from 25 per cent acetic acid solution. This was converted to the hydrochloride; the purine was precipitated with copper and bisulfite, and the cuprous precipitate was decomposed with HCl and H<sub>2</sub>S and the excess of HCl and H<sub>2</sub>S was removed by evaporation. The adenine hydrochloride then was dissolved in hot water with the aid of a slight excess of ammonia, and the free base was precipitated by

TABLE II

*Chromogenic Substances in Adenine and Hypoxanthine Preparations*

Each substance was added in the amount indicated to a standard solution of guanine hydrochloride. The total color then was determined, and the color equivalent of the added substance was estimated by difference, and calculated as micromoles and as moles per cent of guanine.

Substance taken		Chromogenic material found	
	<i>micromoles</i>	<i>micromoles</i>	<i>per cent</i>
Adenine sulfate, Sample I, Eastman	2.86	0.044	1.5
	7.14	0.075	1.05
	14.3	0.128	0.90
	28.6	0.263	0.92
Adenine sulfate, Sample II, Eastman	2.86	0.008	0.28
	7.14	0.030	0.42
	14.3	0.075	0.53
	28.6	0.141	0.49
Hypoxanthine, laboratory Preparation A	1.79	0.000	0.0
	3.57	0.069	1.9
	8.9	0.094	1.1

passing a stream of carbon dioxide through the solution. The adenine so obtained was recrystallized twice from hot water and dried at 105°.

*Hypoxanthine*—Adenine sulfate was deaminized according to the method of Kossel (13). The hypoxanthine was precipitated with silver nitrate, and the silver nitrate compound was recrystallized from 3 N nitric acid. This substance was decomposed with H<sub>2</sub>S. After filtration and removal of the excess H<sub>2</sub>S by boiling, ammonia water was added to slight excess, and boiling was continued until the odor of ammonia was faint. The hypoxanthine crystallized

on cooling. It was recrystallized three times from hot water and dried at 105°.

*Estimation of Guanine and Xanthine in Tissue Extracts*

In order to apply the phenol reagent method for guanine and xanthine to tissues or tissue extracts, necessary preliminaries are, of course, extraction and liberation of the purines and separation of the purines from other chromogens. No fixed analytical scheme which will apply to all situations can be given; the choice and allocation of the various steps will depend on whether one is interested in nucleic acid or extractable purine, and whether the latter is in nucleotide, nucleoside, or free purine form. However, it may be of value to give some suggestions and examples of the way the method may be applied to the estimation of guanine and xanthine in trichloroacetic acid extracts of tissues.

For the precipitation of free purines, with the object of recovering the precipitated material, the copper-bisulfite method of Krüger and Schmid (14) is the procedure of choice, for reasons which have been discussed elsewhere (15). When this method is applied to trichloroacetic acid extracts of tissue, the trichloroacetic acid must be nearly completely removed, for even small amounts of the acid prevent the precipitation of the cuprous purine complex. The hydrolysis of trichloroacetic acid is best carried out in acid solution in which the chief products of the hydrolysis,<sup>1</sup> CO<sub>2</sub> and chloroform, are completely volatile. For complete decomposition, the solution is heated 2 hours at boiling water bath temperature,<sup>1</sup> and H<sub>2</sub>SO<sub>4</sub> equivalent to a final concentration of 1 N is added. This period of hydrolysis insures the liberation of the purines, for both guanine and xanthine are readily released from nucleotide combination during acid hydrolysis (16, 17).

<sup>1</sup> The rate of hydrolysis of trichloroacetic acid in acid solution does not appear to have been reported. In loosely stoppered tubes placed in a boiling water bath, a 5 per cent solution of the acid was found to be 88.4 per cent decomposed after 1 hour, 99.1 per cent after 2 hours. The decomposition is slightly inhibited in the presence of H<sub>2</sub>SO<sub>4</sub>. After 1 hour in the water bath the same trichloroacetic acid solution was 82.1 per cent decomposed in the presence of 0.4 N H<sub>2</sub>SO<sub>4</sub> and 79.2 per cent hydrolyzed when the solution contained H<sub>2</sub>SO<sub>4</sub> equivalent to N concentration. The slight residue of titratable acid which remains after 2 hours hydrolysis is composed mainly (80 per cent or more) of HCl.



From such hydrolysates of trichloroacetic acid extracts of tissues, some non-purine nitrogen sometimes is carried down in the first copper-bisulfite precipitate, especially if the tissue extract has been allowed to decrease in volume during the hydrolysis. This extraneous nitrogen can be removed completely by a second copper-bisulfite precipitation. As a representative example of many such experiments, the following may be cited. To 100 cc. of a 5 per cent trichloroacetic acid extract of cat muscle, representing 9.26 gm. of tissue, 4 cc. of 10 N  $\text{H}_2\text{SO}_4$  were added, and the solution was heated for 2 hours in a boiling water bath. During the period of hydrolysis the solution was allowed to concentrate to a volume of about 40 cc. After neutralization with NaOH, the purines were precipitated with copper and bisulfite and the precipitate decomposed as described below. A Kjeldahl nitrogen determination made on the resulting solution gave the value 59.9 mg. of N per 100 gm. of tissue. An adenine determination (15, 18) on the same solution revealed the presence of 43.0 mg. of adenine N per 100 gm. of tissue. An aliquot of the solution obtained from the copper precipitate was evaporated to dryness and dissolved in water; the purines were reprecipitated with copper and bisulfite, and the precipitate was decomposed as before. This second solution gave a total purine nitrogen value of 44.0 and an adenine value of 42.3 mg. of N per 100 gm. of tissue. A third precipitation resulted in no significant change in the ratio of adenine to total nitrogen, nor in any significant loss in total purine nitrogen. On the basis of such experiments it was concluded that double precipitation of the purines is necessary to exclude any non-protein nitrogenous contamination, and it was adopted as a routine procedure. However, the amount of color given with the phenol reagent by the solutions of the first and second copper precipitates was found to be the same in extracts of a number of tissues. When this has been found to be true of the tissue used, the second precipitation can be omitted in subsequent work.

Cysteine and the reduced form of glutathione react with the phenol reagent, 2 micromoles of the SH compound giving a color equivalent to 1 micromole of guanine or xanthine. As the SH substances form insoluble cuprous precipitates, it was necessary to determine to what extent glutathione and cysteine might interfere with the determination of the chromogenic purines. To solu-

tions containing 3 mg. of adenine N and 1 mg. of guanine N were added 5.65 mg. of cysteine hydrochloride and 11.4 mg. of glutathione, respectively. Analyzed directly, such solutions gave values for guanine of 222 and 227 per cent of the theoretical amount, respectively. Each solution was diluted to 40 cc., 2.5 cc. of 10 N  $\text{H}_2\text{SO}_4$  and 2.5 gm. of trichloroacetic acid were added, and the solution was heated 2 hours in a boiling water bath. While still hot, the solution was neutralized, and the purines were precipitated and reprecipitated by the copper-bisulfite method. The resulting solutions, analyzed by the phenol reagent technique, showed a recovery of 101 and 99 per cent of the guanine taken, compared with a recovery of 97 and 96.5 per cent for similar solutions containing only the adenine and guanine, showing that cysteine and glutathione were nearly completely eliminated by the treatment outlined. The practical absence of glutathione and cysteine from the solutions could be confirmed further by the fact that the solutions gave only a trace of blue color with phospho-18-tungstic acid (uric acid reagent).

Uric acid also gives a color with the phenol reagent and is precipitated by copper and bisulfite. Although uric acid is present in negligible amounts in the tissues of most mammalian species, it should be considered as a possible interfering substance in analyses of tissues from uric acid-forming species. Uric acid may be detected in the solutions from the copper precipitates (after the  $\text{H}_2\text{S}$  is removed) either by means of its reaction with uric acid reagent, or simply by the fact that it reduces the phenol reagent in acid solution. A greenish discoloration of the phenol reagent-purine solution, before the addition of  $\text{Na}_2\text{CO}_3$ , is readily apparent when 0.003 mg. of uric acid is present (equivalent to about 1 per cent of the chromogenic value of the guanine standard). Uric acid can be destroyed before color development, without affecting the purine bases as follows: The solution is boiled to drive off the  $\text{H}_2\text{S}$  and  $\text{HNO}_3$  is added to a concentration of approximately 0.2 N. This solution is heated in a boiling water bath for 10 minutes and then is cooled and used for color development as described previously.

*Reagents—*

Trichloroacetic acid. 50 gm. in 1 liter of water. Cool to  $0^\circ$ .

Sodium bisulfite. Saturated at room temperature in contact with excess solute.

Copper sulfate. 10 gm. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 100 cc. of aqueous solution.

Sulfuric acid. 112 cc. of concentrated sulfuric acid diluted to 1 liter.

Sodium hydroxide. A solution saturated at room temperature.

#### *Procedure*

The tissue is extracted with 10 volumes of an ice-cold 5 per cent solution of trichloroacetic acid, after being minced with a meat chopper or ground with sand. The mixture is allowed to stand 2 to 4 hours at  $5^\circ$  with occasional stirring and then is filtered. To an aliquot of the filtrate in a conical tipped centrifuge tube is added 0.1 volume of 4 N  $\text{H}_2\text{SO}_4$  and the solution is heated 2 hours in a boiling water bath. A drop of phenolphthalein is added, and the solution is neutralized with concentrated NaOH solution, and finally made just acid with dilute  $\text{H}_2\text{SO}_4$ . The centrifuge tube then is returned to the boiling water bath. For each gm. of tissue represented by the aliquot of extract taken, there is added 0.15 cc. of sodium bisulfite solution and 0.1 cc. of copper sulfate solution. The solutions are mixed, and the tube is allowed to remain in the boiling water bath until the precipitate coagulates and begins to turn brown (2 to 3 minutes). If the precipitate does not turn brown, small additional quantities of the reagents may be added, but excessive amounts are strictly to be avoided. When precipitation is complete, the mixture is centrifuged, and the precipitate is washed twice on the centrifuge with hot water. The precipitate is suspended in 3 N HCl, 0.4 cc. being used per gm. of tissue, and the mixture is heated to boiling. About 2 cc. of hot water per gm. of tissue now are added; the tube is placed in a hot water bath, and a rapid stream of  $\text{H}_2\text{S}$  is passed through the mixture for about 3 minutes. The mixture is cooled, transferred to a volumetric flask, diluted to the mark, mixed, and filtered. An aliquot of the filtrate is evaporated to dryness on the water bath by means of a stream of air, with the precaution that the final 0.2 volume is evaporated at a water bath temperature not exceeding  $40^\circ$ . The purine hydrochlorides are dissolved in an amount of water equivalent to about 4 cc. per gm. of tissue, and are precipitated with 0.1 cc. of copper solution and 0.08 cc. of sodium bisulfite solution per gm. of tissue represented. The washing and subsequent manipulations are carried out as in the previous precipitation.

An aliquot part of the purine solution estimated to contain between 0.7 and 2.8 micromoles of chromogenic purine is now transferred to a test-tube calibrated to contain 25.0 cc. This solution is boiled until all the hydrogen sulfide is expelled, and then is used for the colorimetric determination as previously described. The content of guanine in the tissue is calculated on the assumption that there is a uniform distribution between the tissue residue and extracting medium and a tissue water content of 80 per cent.

In Table III are given the results of a number of representative determinations of the extractable guanine and xanthine content

TABLE III  
*Extractable Guanine and Xanthine of Tissues*

Experiment No.	Tissue	Chromogenic purine <i>mm per kg. tissue</i>
1	Cat kidney	0.772
2	" liver	0.572
3	" heart, 2 hrs. post mortem	0.272
4	" skeletal muscle	0.076
5	Rabbit liver	0.626
6	" kidney	0.616
7	Mouse skeletal muscle	0.047
8	Rat skeletal muscle	0.107
9	Mouse Sarcoma 180	0.343
10	Carcinoma 256	0.037
11	Rat muscle*	1.02
12	" Carcinoma 256*	1.13
13	Mouse Sarcoma 180, 2 hrs. at 38°	1.94

\* 2 hours at 38° with 2 volumes of 2 per cent NaHCO<sub>3</sub>.

of tissues, made in the manner described. The data for skeletal muscle may be compared with the report of Dmochowski and coworkers (19) who found about 1 mg. per cent of guanine (0.066 mm per kilo) in rabbit muscle extract and the report of Ostern (20) who found 0.65 and 0.93 mg. per cent of extractable guanine in the same tissue. Data for the extractable guanine content of other tissues are lacking in the literature. Presumably nearly all the chromogenic purine of extracts of fresh tissues may be considered to be guanine, probably present in the tissues as guanylic acid. In this connection it is interesting that the isolation

of guanylic acid from liver tissue has been reported (21) but there are no data in the literature concerning its presence in kidney, which apparently is quite as rich a source. The extractable guanine of the liver and kidney of two species (cat and rabbit) is about 10 times that of the muscle.

During postmortem autolysis the extractable chromogenic purine of muscle increases (Experiment 11, Table III). The greater part of this material is xanthine, as shown by its failure to form precipitates with ammonia and picrate under conditions which precipitate guanine.

The determinations of guanine in tumor tissue (Experiments 9, 10, 12, 13, Table III) show that the content of extractable guanine of neoplasms covers much the same range as that of normal tissues. Thus Sarcoma 180, with a total acid-soluble purine content of 22 mg. per 100 gm., contains about half as much chromogenic purine as liver or kidney, in which similar amounts of total extractable purine occur, and 3 to 5 times as much as muscle, which has approximately 45 mg. of total extractable purine per 100 gm. It is interesting in this connection to note that Sarcoma 180 contains a small but appreciable guanylic acid deaminase activity.<sup>2</sup>

Carcinoma 256 was found to have a very low acid-soluble purine content (about 10 mg. per 100 gm. of tissue) compared with muscle, organs, and Sarcoma 180. As in muscle, the extractable chromogenic purine of this tissue represents only a small fraction of the total purine.

The relatively large increase in the acid-soluble chromogenic purine of tumor tissue during autolysis presumably has its origin, at least in part, in nuclear purine. This is borne out by the finding that the total extractable purine increases (despite nearly complete deamination of the adenine) during incubation. Moreover, after autolysis of Sarcoma 180 (Experiment 13, Table III) the extract yielded more crystalline guanine picrate than did similar extracts of fresh tissue.

#### DISCUSSION

The method described herein, in common with all oxidative procedures, is limited in its applications, because the reagent

<sup>2</sup> Unpublished experiments with Lewis M. Kane.

employed is relatively unspecific. The purines can be separated from other interfering substances, but there is as yet no satisfactory means of separating small amounts of guanine and xanthine. This limitation is perhaps not as severe as might be apparent, for in many purine studies only one or the other of these substances is likely to be present. The method thus is applicable to studies of the purine composition of fresh tissues or tissue extracts in which guanine and adenine are the only purines present, and to the analysis of mixtures of hypoxanthine and xanthine, such as might be found in xanthine oxidase studies. Because guanine and xanthine differ in the number of nitrogen atoms per molecule, the composition of mixtures of these two substances can be ascertained indirectly with an error of 5 to 10 per cent by calculation from the data for nitrogen content and color production of the given solution.

#### SUMMARY

Guanine and xanthine react with the phenol reagent to give a blue color, the intensity of which is proportional to the number of molecules of chromogenic substance present in the solution. Adenine and hypoxanthine, when pure, do not react with the reagent. The reaction, therefore, can be applied to the determination of guanine or xanthine in a variety of situations, some of which have been suggested.

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## THE ISOLATION OF 17-HYDROXYPROGESTERONE FROM THE ADRENAL GLAND

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During the past 6 years twenty-three steroids have been isolated from extracts of the adrenal glands of cattle. Among them is a series of four compounds which are allopregnane derivatives having 3 atoms of oxygen in the molecule, the so called  $C_{21}O_3$  series of adrenal steroids. Reichstein and his coworkers showed these four compounds to be allopregnane- $3\beta,17\beta$ -diol-20-one, the two stereoisomeric allopregnane- $3\beta,17\beta,20$ -triols, and  $\Delta^4$ -pregnene-21-ol-3,20-dione (1). We have isolated a fifth member of this series from adrenal extracts. On examination it was found to be the hitherto unknown steroid,  $\Delta^4$ -pregnene-17-ol-3,20-dione or 17-hydroxyprogesterone (I), a position isomer of desoxycorticosterone.

The compound was obtained in small amount from a ketonic fraction after the more reactive alcohols were separated with succinic anhydride. It crystallized from a concentrated acetone solution and was readily secured in pure form by repeated crystallization from acetone and ethanol. It melted at  $212-215^\circ$ . The elementary analyses and molecular weight data agreed with the formula  $C_{21}H_{30}O_3$ . Its specific rotation in chloroform was  $[\alpha]_D^{27} = +102^\circ \pm 3^\circ$ . A strong selective absorption in the ultraviolet<sup>1</sup> region at  $242\text{ m}\mu$  ( $\epsilon_{\text{max.}} = 18,600$ ) indicated the presence of an  $\alpha,\beta$ -unsaturated ketone group of the cholestenone type (Fig. 1). The compound did not react with acetic anhydride in pyridine at room temperature. Two carbonyl groups were shown to be present by the preparation of a disemicarbazone and a dioxime, both of which on analysis appeared to be the respective derivatives of a compound having the molecular composition  $C_{21}H_{30}O_3$ . Since

<sup>1</sup> The observations on the ultraviolet absorption were made by Dr. D. T. Ewing, Michigan State College, East Lansing.



the 3rd oxygen atom was relatively inert, it appeared by analogy with the known adrenal steroids that the new compound might be either 11-hydroxy-, 11-keto-, or 17-hydroxyprogesterone. Oxidation with chromic acid in glacial acetic acid at room temperature yielded a crystalline neutral oxidation product which melted at 168-169° and which was found to be identical with  $\Delta^4$ -androstenedione-3,17 (II) by analysis and mixture melting point. The structure of the new compound was thus demonstrated to be 17-hydroxyprogesterone. No direct evidence is available on the steric configuration around carbon atom 17. By analogy with the

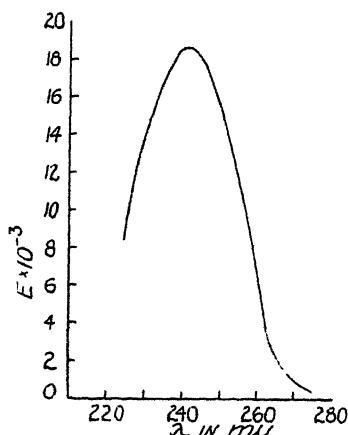
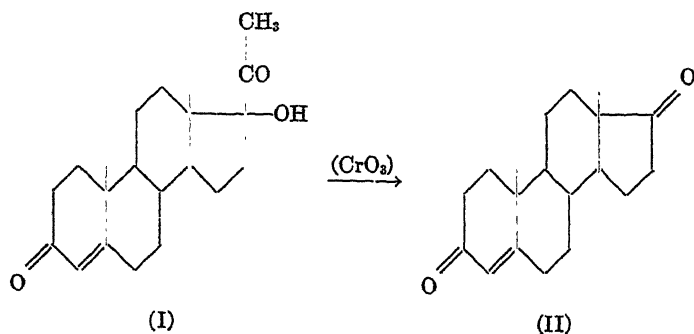


FIG. 1. Ultraviolet absorption of 17-hydroxyprogesterone in ethanol

known adrenal steroids which have been shown by Reichstein to have the  $\beta$  configuration (2-4) the compound is most likely



17- $\beta$ -hydroxyprogesterone. Neither of the 17-hydroxyprogesterones has been prepared as yet by synthetic means. The compound to which Ruzicka and Meldahl (5) ascribed the structure of 17- $\alpha$ -hydroxyprogesterone was later recognized as a product of rearrangement which no longer possessed the pregnane carbon skeleton (6, 7).

Our sample of 17-hydroxyprogesterone failed to elicit any progestational reaction in a series of three rabbits which received intramuscularly 1.9, 2.5, and 5.0 mg. doses, respectively. A slightly modified Clauberg technique was employed.<sup>2</sup> Although it cannot be concluded from these tests, which are necessarily

TABLE I

*Effect of 17-Hydroxyprogesterone and Adrenosterone on Weight of Seminal Vesicles and Prostate of Castrated Rats*

Compound	No. of rats	Body weight	Total dose	Weight of seminal vesicles	Weight of prostate
		gm.	mg.	mg.	mg.
17-Hydroxyprogesterone. . . .	6	43	1.0	10	23
Adrenosterone.....	5	51	0.75	8.5	19
Androsterone.....	5	51	0.75	9	20
None.....	50	50		6.5	9

The dose was evenly divided in six injections administered subcutaneously in 0.25 cc. of peanut oil. The rats were castrated at 25 days of age and injected daily for the following 6 days. The animals were killed on the 7th day after castration. The seminal vesicles and the ventral lobe of the prostate were dissected out and weighed.

limited by the quantities of the compound available, that 17-hydroxyprogesterone has no progestational activity, it is clearly less active than 21-hydroxyprogesterone. The latter compound at a dose level of 5 mg. was strongly active. Progesterone itself gave a comparable reaction at a dose level of 0.5 mg. As might be expected, 17-hydroxyprogesterone was found inactive when tested for cortical hormone activity. It was administered subcutaneously in a daily dose of 0.25 mg. to a series of six 30 day-old rats, following adrenalectomy. They survived an average of 6 days, just as did untreated, adrenalectomized, control animals. Cor-

<sup>2</sup> We are indebted to Dr. D. A. McGinty of this laboratory for the assay of progestational and androgenic activities.

ticosterone was active at a dose level of 0.1 mg. per rat per day. The compound was also entirely inactive in the acute muscle work test of Ingle (8) in a series of four animals in doses of 1.0, 2.0, 2.0, and 3.0 mg., respectively.<sup>3</sup> When examined<sup>2</sup> for androgenic activity in the castrate rat, 17-hydroxyprogesterone exhibited an activity comparable to androsterone and adrenosterone, as is evident from the data summarized in Table I. No androgenic activity was observed in the capon<sup>4</sup> when tested at a level of 200  $\gamma$  per day. The standard daily dose of androsterone in the capon assay is 100  $\gamma$  (9). Limited supplies of the compound prevented further tests at higher levels. The results on the castrate rats, although admittedly having limited quantitative significance, would indicate that 17-hydroxyprogesterone may play a rôle along with adrenosterone (1) in the adrenal-gonad relationship.

#### EXPERIMENTAL<sup>5</sup>

The adrenal extract was prepared by the methods of Swingle and Pffner (10) and further fractionated into the so called first and second ether concentrates (11). The first ether concentrate was separated into ketonic and non-ketonic fractions by means of Girard's Reagent T (betaine hydrazide hydrochloride). The ketonic complex was fractionally hydrolyzed, a fractionation procedure first employed by Reichstein (12). The ketonic fraction liberated between pH 6 and pH 4 weighed 9.9 gm. from approximately 3 tons of beef adrenal glands. The highly pigmented sirup was dissolved in 320 cc. of methyl alcohol and 80 cc. of water were added, containing 8 gm. of  $\text{KHCO}_3$ . The mixture was refluxed for 1 hour, the methyl alcohol distilled off under reduced pressure, and the saponification mixture extracted six times with 500 cc. portions of ether. The ether-soluble fraction was a pale yellow sirup weighing 4.3 gm. It was dissolved in 30 cc. of py-

<sup>3</sup> We wish to thank Dr. D. J. Ingle for his kindness in testing this compound.

<sup>4</sup> The capon assay was made at the University of Chicago through the courtesy of Professor F. C. Koch.

<sup>5</sup> All melting points were determined in a Berl block and are uncorrected. The microanalyses were made by Mr. Clark Chamberlain of this laboratory. For physiological assays the compounds were dissolved in peanut oil except in the case of the muscle work test by Dr. D. J. Ingle, when sesame oil was employed.

ridine; 5.5 gm. of succinic anhydride were added and the mixture warmed gently at 50° until the anhydride had dissolved. The mixture was allowed to stand overnight, the bulk of the pyridine removed by distillation under reduced pressure, the residue taken up in ether, and the remaining pyridine removed by washing with small quantities of dilute hydrochloric acid. The half esters were separated from the neutral fraction in the usual manner with half saturated sodium carbonate. The ether solution of the neutral fraction was dried with anhydrous sodium sulfate and the ether removed. The pale yellow residue weighed 1.4 gm. It was dissolved in 2.5 cc. of acetone. A crystalline deposit started to form promptly. After the solution had stood several days in the refrigerator, the crystalline fraction was filtered off and washed with cold acetone. It was dried and weighed 264 mg. The crude crystals melted at 190–195°, with softening from about 185°. Three recrystallizations from acetone and two from ethanol yielded 60 mg. of thin platelets melting at 212–215°. The melting point was not changed on further recrystallization. It is somewhat dependent on the rate of heating. Another 100 mg. of the same compound, m.p. 210–212°, was obtained when the mother liquors were worked up. The compound is readily soluble in chloroform but insoluble in ether. It crystallizes from ethyl acetate in platelets. It does not precipitate with digitonin either in 80 per cent ethyl alcohol or 50 per cent methyl alcohol. The specific rotation is  $[\alpha]_D^{27} = +102^\circ \pm 3^\circ$  ( $c = 1.56$  in chloroform). The compound was dried for analysis *in vacuo* at 110° for 3 hours.

*Analysis*— $C_{21}H_{30}O_8$ . Calculated. C 76.3, H 9.2, mol. wt. 330

Found. " 76.0, " 9.3, " " 363

" 76.0, " 9.1

27 mg. of the compound were allowed to stand overnight in 1 cc. of acetic anhydride and 0.7 cc. of pyridine. The mixture was distilled to dryness and the residue recrystallized twice from ethanol. The product weighing 23 mg. melted at 211–213°, had the same crystal form as the starting material, and failed to depress the melting point of the original compound. It was dried for analysis *in vacuo* at 110° for 3 hours.

*Analysis*— $C_{21}H_{30}O_8$ . Calculated. C 76.3, H 9.2

Found. " 76.0, " 9.4

" 75.8, " 9.2

*Disemicarbazone*—15 mg. of the compound were refluxed for 30 minutes in 1 cc. of ethanol with 30 mg. of semicarbazide acetate. On dilution with water the product separated promptly in crystalline form. It was collected, washed thoroughly with water and alcohol, and dried. The product weighed 19.8 mg. It did not melt below 360°. It darkened at about 240° and sintered markedly at 280–290°. Because of its extreme insolubility in ethanol it was not recrystallized for analysis. The compound was dried for analysis at 110° *in vacuo*.

*Analysis*— $C_{23}H_{30}O_2N_6$ . Calculated. C 62.1, H 8.2, N 18.9  
Found. " 61.3, " 8.1, " 18.8  
" 61.4, " 8.1

*Dioxime*—14 mg. of the compound were refluxed for 4 hours in 3 cc. of 95 per cent alcohol with 100 mg. of hydroxylamine acetate. The mixture was boiled down to 1 cc. and 2 cc. of water were added. The product separated promptly. It was filtered off and recrystallized from 50 per cent ethanol. It separated in thin plates which melted with decomposition at 250–251°, with sintering from about 240°. The compound weighing 9 mg. was dried for analysis at 110° *in vacuo* for 2 hours.

*Analysis*— $C_{21}H_{32}O_3N_2$ . Calculated, N 7.8; found, N 7.9

*Oxidation with Chromic Acid*—41 mg. of the compound were dissolved in 1 cc. of glacial acetic acid and 25 mg. of chromic acid in 0.5 cc. of 90 per cent acetic acid added. The mixture was allowed to stand overnight and the excess chromic acid was reduced with sodium sulfite. After the mixture was distilled to dryness under reduced pressure at 50°, the residue was taken up in water and ether and the ether-soluble fraction freed of acids with aqueous sodium carbonate. The dried neutral ether-soluble fraction weighed 32 mg. It was taken up in 2 cc. of benzene and filtered through 200 mg. of aluminum oxide. The filter was washed well with benzene, the filtrate evaporated to dryness, and the residue recrystallized twice from dilute ethanol, yielding 13 mg. of rosettes of microscopic needles, m.p. 168–169°. A mixture melting point with  $\Delta^4$ -androstenedione-3,17, m.p. 167–168°, showed no depression. The product was dried for analysis at 80° *in vacuo* for 4 hours.

*Analysis*— $C_{19}H_{26}O_2$ . Calculated. C 79.7, H 9.2  
Found. " 79.2, " 9.6

## SUMMARY

The isolation of a new steroid ketone from the adrenal glands of cattle is described. Its structure is shown to be that of a 17-hydroxyprogesterone.

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## THE GROWTH FACTORS IN CARTILAGE FOR THE CHICK\*

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Previous work (1, 2) with purified rations for chicks showed that cartilage, kidney, and certain other natural materials contained a factor (or factors) that was essential for normal growth. The properties and distribution of the factor suggested that it was distinct from those known to be essential in chick nutrition. We were aware that our basal ration was low in arginine, since Arnold, Kline, Elvehjem, and Hart (3) had shown that 18 per cent of casein did not supply an optimum amount. We did not consider that this was a limiting deficiency, however, since edestin showed only slight activity. Chondroitin also failed to stimulate growth, although Bird, Oleson, Elvehjem, and Hart (4) in 1938 had shown it to have a slight growth-promoting activity. Following the demonstration by Almquist and coworkers (5, 6) of the essential nature of glycine and chondroitin or certain other carbohydrates in chick nutrition, we were able to explain the multiple deficiency existing in our ration. In this paper we wish to present our data which confirm and extend the observation of the California group.

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The yeast used in these experiments was supplied by Dr. Harold F. Levine of the Pabst Brewing Company, Milwaukee; the haliver oil by Dr. C. Nielson of the Abbott Laboratories, North Chicago; the liver extract and chondroitin by Dr. David Klein of The Wilson Laboratories, Chicago; and the soy bean oil by the Allied Mills, Peoria, Illinois.



## EXPERIMENTAL

Day-old white Leghorn chicks were used throughout and handled as previously described (2). The experimental ration used in most of the studies was Ration 469 which has the following percentage composition: dextrin 64, crude casein 18, Salts 4.5 (7), soy bean oil 5, liver extract<sup>1</sup> 3, and brewers' yeast 5. All chicks were dosed weekly with haliver oil containing additional vitamin D. Supplements to the ration were made at the expense of the dextrin.

The growth obtained with this ration and various supplements is shown in Table I. The weight of the chicks is expressed as per cent of the weight of the basal group at 4 weeks of age, since this allows a better comparison of the groups when experiments are conducted over a considerable period and the source of chicks is variable. The chicks upon the basal ration usually weigh between 80 and 90 gm. at 4 weeks.

Very high levels of fish-meal or casein gave a rather marked growth stimulation, although it did not equal that obtained with 15 per cent of cartilage. Arginine alone gave consistent though slight gains over the basal weight, while glycine alone actually inhibited growth. Chondroitin alone gave no response. The combination of all three of these supplements is necessary to obtain satisfactory growth, since no two gave greater growth than that obtained with arginine alone. 5 per cent of chondroitin was necessary for maximum growth but 2 per cent of glycine was not superior to 1 per cent. Arabinose and xylose at levels of 1 per cent were as effective as chondroitin at 5 per cent.

Gelatin at a 5 per cent level has consistently given better growth than arginine and glycine together. Also gelatin plus chondroitin gave more rapid growth than the combination of arginine, glycine, and chondroitin, although glycine and arginine have been fed at approximately the level supplied by 5 per cent gelatin. We should also like to point out a marked difference in various gelatins. Over a year ago and at various times since then we have fed one brand of gelatin with poor results. Consistent results have been obtained with another gelatin used in the experiments reported in this paper.

Raising the level of casein to 30 per cent supplies sufficient

<sup>1</sup> Wilson Fraction D.

TABLE I  
*Effect of Various Supplements on Growth and Gizzard Erosion*

Supplement to Ration 469	No. of groups	Basal weight at 4 wks.	Gizzard erosion*			
			Very slight or none	Slight	Marked	Severe
		<i>per cent</i>				
None.....	6	100		4	8	7
40% casein.....	2	153	5	2	1	2
40% washed fish-meal.....	1	162				
15% cartilage.....	5	176	14	4	2	
0.5-1.5% arginine.....	3	122		8	4	4
1.0% glycine.....	2	88	1	2	5	1
3-10% chondroitin.....	3	98	5	5	2	
1% glycine +0.5% arginine.....	3	125	1	5	2	3
1-2% glycine + 5-10% chondroitin.....	4	119	4	13	6	4
0.5% arginine + 5% chondroitin.....	1	117		3	1	2
1% glycine +0.5% arginine + 3% chondroitin.....	3	144	8	9	2	
1% glycine +0.5% arginine +5% chondroitin.....	3	160	5	8	2	2
1% glycine + 0.5% arginine + 10% chondroitin.....	2	160	11	5		2
2% glycine + 0.5% arginine + 5% chondroitin.....	1	157		2	2	
1% glycine + 0.5% arginine + 1% arabinose.....	2	153	4	2	4	
1% glycine + 0.5% arginine + 1% xylose.....	2	154	1	4	4	2
5% gelatin.....	3	145		6	10	2
5% " + 3-5% chondroitin.....	3	188	16	5	1	
12% casein.....	3	117	1	5	7	3
12% " + 5% chondroitin.....	1	167	1	3	1	
12% " + 5% " + 1% glycine.....	1	175	3	3		
12% casein + 1% glycine + 1% arabinose.....	2	155	3	6	2	
12% casein + 1% glycine + 1% xylose.....	2	111		2	3	

\* Some groups were not graded and no autopsy was performed on chicks which died before 4 weeks.

arginine (8). Apparently some glycine is also supplied, since chondroitin alone gives a marked response and only a slight addi-

tional response is obtained by further supplementation with glycine. In two trials with this ration arabinose gave much better growth than the same level of xylose.

The gizzards of most of the chicks have been graded for gizzard erosion as a matter of routine. These data are also presented in Table I. Although there appears to be some slight improvement with any increase in growth, the action of chondroitin seems most specific. Especially notable is the improvement upon the addition of chondroitin to the basal ration plus gelatin.

TABLE II  
*Response Obtained with Various Supplements on Modified Ration*

Supplement to Ration 469 + 5 per cent additional yeast and 0.05 per cent choline	Basal weight at 4 wks.	Gizzard erosion			
		Very slight or none	Slight	Marked	Severe
	<i>per cent</i>				
None .....	100		2	5	4
0.5% arginine. ....	132		1	3	1
1% glycine. ....	103		1	2	3
10% chondroitin ..	81		4	2	
10% " + 1% glycine...	124	3	7	2	
10% " + 0.5-1% arginine	170	7	1	2	1
1% glycine + 0.5-1% arginine.	211	2	4	3	2
1% " + 0.5-1% " + 5% chondroitin .....	213		3	2	
1% glycine + 0.5-1% arginine + 10% chondroitin . . . .	207	11	1		
15% cartilage. . . . .	244	6			

Recent studies with the basal ration supplemented with additional yeast and choline are presented in Table II. These results are in accord with those presented above, except that chondroitin showed no growth response above that obtained with arginine and glycine. Apparently the additional yeast supplies material which can replace chondroitin. The data show that very high levels of chondroitin are required to prevent gizzard erosion. Since the additional chondroitin gave no growth response, it is doubtful whether the improvement can be explained by an increased consumption of constituents in the basal ration. Cartilage gives better growth than the combination of supplements and also prevents any serious gizzard erosion.

## DISCUSSION

The data presented confirm the observation of Almquist, Stokstad, Mecchi, and Manning (5) that chicks require glycine and chondroitin or certain carbohydrates for normal growth. The activity of chondroitin in the treatment of stomach ulcers in humans (9) led Bird, Oleson, Elvehjem, and Hart (4) to an examination of its effect upon gizzard erosion. They concluded that gizzard erosion was markedly reduced. It is also significant that chicks receiving chondroitin showed a slight growth response in practically every case. A similar action for aldobionic acid was also described. Later Crandall *et al.* (10) suggested that chondroitin gave slightly greater gains under certain conditions. In view of the large number of deficiencies present in the basal ration used by Bird and associates it is not surprising that only slight gains were noted, although the results are clear in the light of the recent work of Almquist *et al.* (5) and the results presented in this paper.

Arnold, Kline, Elvehjem, and Hart (3) originally demonstrated the very high arginine requirement of the chick and Klose, Stokstad, and Almquist (8) later showed that 30 per cent of casein is necessary to supply adequate amounts. However, the difficulty of adding arginine without contamination of the basal ration prevented us from supplying additional arginine in the original work. Also we felt that in rations containing 18 per cent of casein the arginine would probably not be limiting. The results presented here show that arginine was a limiting factor. Since no significant increase in growth was attained with either glycine or chondroitin until arginine was added, these results further emphasize the dangers in the use of deficient diets, even though the requirement of a factor may be partially satisfied.

The specificity of cholic acid and other bile acids in the prevention of gizzard erosion has been further emphasized in a recent paper by Almquist and Mecchi (11). We have also found cholic acid to be highly effective and certainly the most active material we have fed. However, it seems doubtful that cholic acid is the material present in natural sources which prevents gizzard erosion. It seems more likely that there is some metabolic relationship, although the nature of this is not clear, since Almquist has shown that actual contact of the bile acid with the gizzard lining is neces-

sary and that the output of cholic acid by the liver is not necessarily related to anti-gizzard erosion activity. We feel that a significant effect is produced when high levels of chondroitin are fed although the product is not pure. Almquist and Mecchi (11) have recently reported a slight improvement on rations containing 5 per cent chondroitin.

In a previous paper (7) we pointed out an obvious difference in the choline content of the yeast used in our laboratory and that used by Almquist and coworkers. Here again it is worth while to note that the rations used by the California workers to demonstrate the growth-promoting action of chondroitin contain 10 per cent of yeast, while we find that chondroitin gives no significant response when the ration contains 10 per cent of yeast. Such differences no doubt account for many apparent discrepancies in the literature.

#### SUMMARY

1. Glycine and chondroitin (or certain pentoses) fed together with arginine stimulated the rate of growth of chicks on simplified rations. Neither glycine nor chondroitin stimulated growth when used as a single supplement. Arginine alone gave but a mild effect. These rations contained 18 per cent casein and 5 per cent of yeast.

2. When arginine is combined with glycine and chondroitin, the weight reached is somewhat less than that obtained with cartilage as the supplement.

3. With the level of casein at 30 per cent chondroitin alone gave a marked growth response. The further addition of glycine resulted in slightly better growth. These results confirm the work of Almquist, Stokstad, Mecchi, and Manning.

4. When the ration contained 10 per cent of yeast, glycine and arginine as supplements gave good growth which was not improved by the addition of chondroitin.

5. Cartilage and high levels of chondroitin showed considerable anti-gizzard erosion activity.

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## INHIBITION OF THE CONJUGATION OF SULFANILAMIDE

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Acetylation is involved in the detoxication of amino groups in the animal organism. Two well known examples of this are the acetylation of the amino group of *p*-aminobenzoic acid and the acetylation of cysteine in the formation of mercapturic acid, the acetic acid being an intermediary product in metabolism.

Hensel (1) in 1915 demonstrated increased acetylation resulting from concomitant administration of acetate. Harrow *et al.* (2) subsequently reported that acetylaminobenzoic acid output was increased by feeding a number of substances with the aminobenzoic acid. Ethyl alcohol, tyrosine, alanine, and isoleucine gave an increase of 207, 188, 27, and 12 per cent respectively. Stimulation of general carbohydrate metabolism may have been a factor. The results of Hensel (1) had shown that the amount of acetylaminobenzoic acid recovered in the urine of rabbits after being fed *p*-aminobenzoic acid is increased by the simultaneous administration of acetic acid, pyruvic acid, and ethyl acetoacetate. Harrow and coworkers (3) continued their studies of acetylation and demonstrated that the rabbit acetylates *p*-aminobenzoic acid to the extent of about 25 per cent after doses of 1 gm. The output is increased by the injection of insulin but is not increased by the injection of insulin and glutathione. This effect of glutathione was attributed to its inhibiting action upon the activity of insulin *in vitro*. These workers suggested that insulin increased acetylation by increased mobilization of sugar in the organism.

Klein and Harris (4) studied the acetylation of sulfanilamide *in vitro*. They showed that acetylation of this compound can occur in slices of liver and that the factor limiting the reaction in speed was the rate of acetate production by the tissue. Addition



of acetate increased the amount of conjugation, as did the addition of substances giving rise to acetate in the tissue. Pyruvate, lactate, and acetoacetate had a variable effect in increasing conjugation, but acetate was constant in its effect. The acetylation mechanism occurred only in intact liver cells; it was not reversible; it was inhibited by high concentrations of iodoacetamide and arsenious oxide; it was only slightly inhibited by anaerobiosis; and it varied greatly in animals of the same species.

James (5) observed a significant decrease in the toxicity of both sulfanilamide and sulfapyridine when sodium acetate was given at the same time. He suggested that sulfanilamide killed by a sudden withdrawal of acetate precursors from the body.

In the course of an investigation on the detoxication of sulfanilamide, an attempt was made to determine the mode of action of physiological detoxifying agents in protecting against toxic doses of sulfanilamide. This immediately led to a determination of the acetylation mechanisms, with the results herein reported.

#### *Experimental Technique and Results*

By the technique of Bratton and Marshall (6) for the determination of sulfanilamide, free and combined sulfanilamide was determined in the blood of rats following dosages of sulfanilamide with and without the concomitant administration of detoxifying chemicals; *e.g.*, cystine, glucuronic acid, ascorbic acid, and glycine. Experiments with sulfapyridine and sulfathiazole were abandoned for the present, as methods for the determination of these compounds in the free and acetylated forms proved unreliable in our hands.

In the first experiments, 50 rats were given sulfanilamide at a level of 2 mg. per gm. At the end of 3 hours, the animals were killed and blood obtained for analysis. A second set of rats received in addition to the sulfanilamide a mixture of glycine, ascorbic acid, glucuronic acid, and cystine at a level of 2 mg. per gm. of rat. The average value expressing percentage conjugation of total sulfanilamide in the group receiving sulfanilamide alone was 10.1 per cent. The average value expressing percentage conjugation of total sulfanilamide in the set receiving sulfanilamide and the detoxifying chemicals was 0.9 per cent. This would represent an almost complete inhibition of acetylation by the detoxifying chemicals. A second group was arranged to deter-

mine the effect of time interval on the degree of acetylation observed. Dosage levels of sulfanilamide and of detoxifying chemicals were the same.

Table I shows that total sulfanilamide is essentially unaltered at any time interval, that acetylation is blocked, and that approximately 3 hours elapse before acetylation occurs to a significant extent in the controls receiving no detoxifying chemicals.

Throughout the entire range of time covered in this experiment, from 1 to 24 hours, the blockage of acetylation is evident. The

TABLE I

*Effect of Detoxifying Chemicals on Acetylation of Sulfanilamide in Rats*

The values, in mg. per 100 cc. of blood, are averages for four rats.

Time interval	Sulfanilamide, 2 gm. per kilo •							
	No detoxifying chemicals				Detoxifying chemicals (cystine, glycine, ascorbic acid, glucuronic acid), 2 gm. per kilo			
	Total sulfanilamide	Free sulfanilamide	Conjugated sulfanilamide	Per cent conjugated of total	Total sulfanilamide	Free sulfanilamide	Conjugated sulfanilamide	Per cent conjugated of total
<i>hrs.</i>								
1	41.0	40.0	1.0	2.44	46.6	47.7	0.0	0.0
2	37.2	37.2	0.0	0.0	44.8	44.8	0.0	0.0
3	35.5	34.6	0.6	2.53	33.6	33.6	0.0	0.0
4	41.0	35.2	5.8	14.10	75.0	75.2	0.0	0.0
5	64.0	56.0	8.0	12.5	35.7	35.7	0.0	0.0
7	58.6	54.5	4.1	7.0	38.3	37.8	0.5	1.3
9	51.6	49.6	2.0	3.9	44.0	44.5	0.0	0.0
24	33.0	25.6	7.4	22.4	49.4	49.0	0.4	0.8

detoxifying chemicals used were selected because this combination had the greatest effect in decreasing the acute toxicity of sulfanilamide.<sup>1</sup>

Finally, each of the components of the detoxifying mixture was tested individually for effect in blocking the acetylation of sulfanilamide. Twenty-four rats were used in each of the five series. Ascorbic acid, glycine, and cystine had no effect. The entire action was due to the glucuronic acid. The values are indistinguishable from those presented in Table I. Acetylation at 24 hours is completely blocked by glucuronic acid, whereas the control value without the glucuronic acid averages 15 per cent for

<sup>1</sup> A separate report will be published later.

60 rats. This value expresses the per cent conjugated of the total sulfanilamide.

#### DISCUSSION

There is some evidence in the literature contraindicating the use of acetates with the sulfonamido chemotherapeutic agents. Lehr *et al.* (7) have demonstrated that acetylated sulfathiazole precipitates in massive amounts in the urinary bladder, causing sudden death. The bladder of these animals on occasion becomes completely filled with the white crystalline material. Increased acetylation might aggravate the tendency of sulfathiazole to precipitate in the bladder as the acetylated derivative. Furthermore, Long<sup>2</sup> states that acetylsulfanilamide is more toxic when administered parenterally than is sulfanilamide itself. The decrease of acetylation might well be of therapeutic value in the use of the sulfonamido type of chemotherapeutic agent.

The question remains as to whether or not all ingested sulfanilamide can be accounted for by the excretion of free and acetylated forms or whether it is excreted partly in some other form, such as the glucuronide, etc. The observations of Scudi *et al.* (8) that increased glucuronate excretion follows the administration of sulfapyridine and that (9) hydroxysulfapyridine is present in dog urine following dosages of this compound bear directly on this problem. Scudi (9) isolated a water-soluble glucuronate of hydroxysulfapyridine. Recently, Scudi and Robinson (10) stated that 40 per cent of the so called free sulfapyridine is present as the highly soluble glucuronide. This was established by a study of the ratio of free to acetylsulfapyridine and a qualitative estimation of the urinary hydroxysulfapyridine. They state that the excretion of a part of the drug in a soluble form is important in the etiology of acetylsulfapyridine urolithiasis. Following liver damage induced by phosphorus poisoning, the glucuronic acid output was no longer augmented by the administration of sulfapyridine. Production of the insoluble acetylsulfapyridine was not depressed. From the work of James (11) who isolated *p*-N-acetylhydroxylaminobenzene-sulfonamide, *p*-hydroxyaminobenzenesulfonic acid, and *p*-aminophenol from the urine of patients treated with sulfanilamide, it is clear that the entire amount of ingested sulfanilamide is not excreted as the free or as the acetylated form.

<sup>2</sup> Personal communication from Dr. Perrin H. Long.

Our observations on the blockage of acetylation would indicate that the usual channel of detoxication is altered by a mass action effect to force conjugation into other channels; *e.g.*, to the glucuronide. Increased acetylation has been demonstrated by Klein and Harris (4), by Hensel (1), and by Harrow *et al.* (3), and this increased acetylation resulted entirely from an increase in the acetate available for the reaction. This would indicate a mass action effect. Here, the animal organism is supplied with large amounts of detoxifying chemicals other than acetate, with the result that conjugation proceeds through other channels. A similar effect of increased amounts of one detoxifying chemical, glycine, was shown by Griffith and Lewis (12), who demonstrated that the rate of hippuric acid formation was directly proportional to the amount of glycine available. This facilitation and inhibition of the formation of conjugation products should be useful in the study of detoxication and intermediary metabolism.

The problem of urolithiasis resulting from therapy with the sulfonamido type of chemotherapeutic agent can be approached from this angle. Furthermore, the chemotherapeutic efficacy of urinary sulfonamido compounds might be increased by the prevention of acetylation.

#### SUMMARY

The conjugation of sulfanilamide in the rat is inhibited by the administration of glucuronic acid. The problem of urolithiasis and chemotherapeutic efficacy of compounds of the sulfonamido type is considered in this connection.

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## INTERRELATIONSHIPS IN THE REACTIONS OF HORSE HEMOGLOBIN

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Blood hemoglobin is a molecule capable of undergoing a great variety of reactions. Owing to its amphoteric properties, it takes part in a complex equilibrium involving protons; by virtue of the iron atoms coordinated with the four hemes it is capable of undergoing reversible oxidation and reduction; and in addition to this it enters into reversible combination with a variety of other molecules, of which from a biological point of view oxygen is the one of greatest interest. It is apparent as a result of the extensive investigations of Pauling and his collaborators (5, 4) on the associated magnetic changes that these last reactions represent combination with the hemes (whether oxidized or reduced). These many reactions, representing as they do different faculties of the hemoglobin molecule, may be expected to show interrelations, and in so far as this is so it should be possible to predict the course of one reaction from a suitable knowledge of others. Some success has already been achieved in this direction. Thus it has been possible to predict with fair success the effect of pH on the oxygen equilibrium from a study of the amphoteric behavior of oxygenated and reduced hemoglobin (7). Also Coryell and Pauling have discussed pH effects involving various hemoglobin derivatives in terms of the acidity of groups associated with the hemes (3).

This approach constitutes a problem in the thermodynamics of the hemoglobin molecule, the results of which should provide a check on the correctness of the diverse sets of experimental data involved in the interrelations. In this paper we shall develop it further in a detailed attempt to see just how well it is in fact possible to interrelate certain of these equilibria quantitatively. We shall limit ourselves to three; namely, those involving oxida-

tion-reduction, combination with oxygen, and the dissociation of protons. There are already available the extensive measurements of Ferry and Green (6) on the oxygen equilibrium, those of Taylor and Hastings (10) on the oxidation-reduction potentials, and those of German and Wyman (7) giving the differential titration data for hemoglobin, Hb, and oxyhemoglobin,  $\text{Hb}(\text{O}_2)_4$ . All that remains therefore in order to provide the necessary experimental basis for our purpose is to carry out the differential titration of ferrihemoglobin,  $\text{Hb}^{++++}$ , against either hemoglobin, Hb, or oxyhemoglobin,  $\text{Hb}(\text{O}_2)_4$ . This has now been done for  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$ , and the results are presented below. Before we proceed to a consideration of them, however, we shall present briefly the basic relations necessary for the analysis. This presentation is in part a restatement in somewhat more general form of relations already developed (7).

Consider a substance M capable of combining with a number of reactants. For simplicity, assume that these are limited to three; namely, X, Y, and Z. Introduce the symbol  $M$  to denote the concentration of the completely uncombined molecules of M, and the symbols  $x$ ,  $y$ , and  $z$  to denote the activities of X, Y, and Z respectively. Assume that in all each mole of M is capable of combining with  $p$ ,  $q$ , and  $r$  moles of X, Y, and Z respectively.

Now let us fix our attention on that class of molecules of M which are combined with just  $s$  molecules of Y, regardless of the amount of X and Z combined. We refer to these by  $\text{MY}_s$  and to their concentration by  $\text{MY}_s$ . Then

$$(1) \quad \text{MY}_s = M \cdot y^s \sum_{i=0}^p \sum_{n=0}^r K_{in} x^i z^n$$

At the same time the amount of X in combination with them, expressed as a concentration, is given by

$$(2) \quad M \cdot y^s \sum_{i=0}^p \sum_{n=0}^r i K_{in} x^i z^n$$

In these two double sums the constants  $K$  are apparent constants, like the acidity constants of Brönsted, since they are based on the use of concentrations in regard to M and its compounds, and since  $x$ ,  $y$ , and  $z$  denote activities. Each  $K$  is itself the sum of

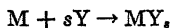
$$\frac{p!}{l!(p-l)!} \cdot \frac{q!}{s!(q-s)!} \cdot \frac{r!}{n!(r-n)!}$$

individual constants, one for each of the microscopically different ways in which combination with X, Y, and Z can occur. Each individual constant is equal to the corresponding thermodynamic constant multiplied by the ratio  $\gamma/\gamma'$  where  $\gamma$  is the activity coefficient of the completely uncombined molecules and  $\gamma'$  is the activity coefficient of the compound. Subject to this convention Equations 1 and 2 are perfectly general and hold whether or not there are interactions between the various combining centers.

We are interested in the amount of X combined per mole of  $MY_s$ , a ratio which we denote by  $X_s$ . It follows from Equations 1 and 2 that

$$(3) \quad X_s = \frac{\sum_{i=0}^p \sum_{n=0}^r l K_{ln} x^i z^n}{\sum_{i=0}^p \sum_{n=0}^r K_{ln} x^i z^n}$$

Now it is to be observed that, if we neglect the possible variation of  $\gamma/\gamma'$  with the activity of X, the numerator of the right-hand member of this equation is the derivative of the denominator with respect to  $\ln x$ . At the same time it may be seen from Equation 1 that the denominator is itself nothing but the constant for the reaction



It is an apparent constant, like the  $K$  values of which it is composed. We shall denote it by  $L_s$ . Consequently we may rewrite Equation 3 as

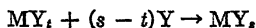
$$(4) \quad X_s = \left( \frac{\partial \ln L_s}{\partial \ln x} \right)_z$$

Equation 4 gives a simple expression for the change of  $L_s$  with  $x$  in terms of  $X_s$ .

If we consider another class of molecules, namely those which are combined with  $t$  molecules of Y, we have a corresponding equation in which  $s$  is replaced by  $t$ . We can combine this with Equation 4 with the result

$$(5) \quad X_s - X_t = \left( \frac{\partial \ln L_{s,t}}{\partial \ln x} \right)_z$$

In this equation  $L_{s,t}$  is the constant for the reaction





Equation 5 expresses in general form the nature of the interrelation between one reaction and another in the case of a complex molecule capable of reacting in a number of different ways.

The relations just outlined are directly applicable to the case of hemoglobin in which we are interested. For this purpose we identify  $x$  with hydrogen ion (proton) activity  $H$ ,  $y$  with the partial pressure of oxygen  $p$ , and  $z$  with an appropriate function of the oxidation-reduction voltage  $E$  (see below). Then  $X$  is to be identified with the number of undissociated protons,  $Y$  with the number of moles of oxygen combined, and  $Z$  with the number of oxidized hemes, all per mole of hemoglobin.

Consider first the oxygen equilibrium. Since there are four hemes in each molecule of hemoglobin, each heme capable of combining with 1 molecule of oxygen, there are five classes of hemoglobin molecule, namely  $Hb$ ,  $HbO_2 \dots Hb(O_2)_4$ , characterized by the number (0, 1 ... 4) of molecules of oxygen combined, and four constants governing the oxygen equilibrium. It is most convenient to choose for these the four constants governing the four equilibria between  $Hb$  and  $HbO_2 \dots Hb(O_2)_4$ . We denote these by  $L_1 \dots L_4$  respectively. Then, from Equation 5

$$(6) \quad X_1 - X_0 = -\left(\frac{\partial \log L_1}{\partial pH}\right)_E \quad X_4 - X_0 = -\left(\frac{\partial \log L_4}{\partial pH}\right)_E$$

If we introduce the symbol  $B$  to denote the amount of base bound per mole of hemoglobin, we may write

$$(7) \quad B_1 - B_0 = -(X_1 - X_0) \dots B_4 - B_0 = -(X_4 - X_0)$$

Now from the basic observation of Ferry and Green that the effect on the oxygen equilibrium curves of changing the pH is the same as changing the scale of  $p$  (partial pressure of oxygen) it can be shown (13) that

$$(8) \quad \frac{\partial \log L_1}{\partial pH} = \frac{1}{2} \frac{\partial \log L_2}{\partial pH} = \frac{1}{3} \frac{\partial \log L_3}{\partial pH} = \frac{1}{4} \frac{\partial \log L_4}{\partial pH} = \left(\frac{\partial \log 1/p}{\partial pH}\right)_E$$

Consequently

$$(9) \quad \left(\frac{\partial \log 1/p}{\partial pH}\right)_E = B_1 - B_0 = \frac{B_2 - B_0}{2} = \frac{B_3 - B_0}{3} = \frac{B_4 - B_0}{4}$$

Since oxidation destroys the power of hemoglobin to combine with oxygen, the experiments involving oxygen are made as far

as possible on pure ferrohemoglobin; *i.e.*, at such a low value of the oxidation-reduction potential  $E$  that  $Z$ , the fraction oxidized, is zero. This simplifies the situation. If we assume that there is no interaction between the various acid groups, and replace  $x$  by  $H$ , it may be seen from Equation 1 and the definition of  $L_4$  that

$$(10) \quad L_4 = k_0 \frac{(1 + H/k_1)(1 + H/k_2) \cdots (1 + H/k_p)}{(1 + H/k'_1)(1 + H/k'_2) \cdots (1 + H/k'_p)}$$

$k_0$  is the apparent constant for the equilibrium between the molecules of Hb from which all possible protons are dissociated and the molecules of  $\text{Hb}(\text{O}_2)_4$  from which likewise all possible protons are dissociated;  $k_1, k_2 \dots k_p$  are the apparent dissociation constants (acidity constants) of the  $p$  acid groups in  $\text{Hb}(\text{O}_2)_4$ ; and  $k'_1, k'_2 \dots k'_p$  are the apparent dissociation constants of the  $p$  acid groups in the Hb.<sup>1</sup> Equations 8 and 10 provide the means of integrating Equation 9 and lead finally to the expression

$$(11) \quad \log (1/p)_T = \frac{1}{4} \log \left[ \frac{(k_1 + H)}{(k'_1 + H)} \cdots \frac{(k_p + H)}{(k'_p + H)} \right] + \text{constant}$$

The  $k$  values in the numerator are for  $\text{Hb}(\text{O}_2)_4$ ; those in the denominator for Hb.

An entirely similar application may be made to the oxidation-reduction equilibrium. Since there are four oxidizable hemes in each molecule of hemoglobin, this equilibrium must depend on four constants, like the oxygen equilibrium. For these it is convenient to choose the constants governing the four equilibria between Hb and  $\text{Hb}^+$ ,  $\text{Hb}^{++}$ ,  $\text{Hb}^{+++}$ , and  $\text{Hb}^{++++}$ , according to the procedure used in discussing the oxygen equilibrium. To these four constants there correspond four voltages,  $E_{0_1} \dots E_{0_4}$ , in terms of which we may discuss the equilibrium. For any reversible oxidation involving  $n$  equivalents which occurs in connection with a standard hydrogen electrode it is customary to write

$$(12) \quad E = E_{0_n} + \frac{RT}{nF} \ln \frac{\text{oxidized}}{\text{reduced}}$$

<sup>1</sup> Formally it is always possible to treat the case as if there were no interaction and express  $L_4$  as in Equation 10, since there are just  $p$  individual groups with constants to be determined, and since  $p$  over-all experimental constants are involved (see Equation 1); but if there were interaction, the required values of  $k$  *might* turn out to be negative, imaginary, or complex.

where  $E$  denotes voltage,  $R$  the gas constant,  $T$  the absolute temperature,  $F$  the Faraday number, oxidized the activity of the oxidized molecules, and reduced that of the reduced molecules. Each of the four  $E_0$  values enters into an equation of this form. Equation 12 is to be compared with the equation for the combination of 1 mole of hemoglobin with  $n$  moles of oxygen; namely,

$$(13) \quad n \ln p = -\ln L_n + \ln \frac{\text{Hb}(\text{O}_2)_n}{\text{Hb}}$$

This is itself simply the logarithmic form of the more familiar equation

$$\frac{\text{Hb}(\text{O}_2)_n}{\text{Hb} \cdot p^n} = L_n$$

Comparison shows that Equations 12 and 13 are formally the same if we identify  $(F/RT) E$  with  $\ln p$  and  $-(nF/RT) E_{0_n}$  with  $\ln L_n$ . The latter identification applies of course to each of the four  $E_0$  values and the corresponding  $L$ . Now the experiments of Taylor and Hastings show that the effect of changing the pH on the curves in which percentage oxidation is plotted against  $E$  is simply to produce a parallel shift along the  $E$  axis. This corresponds exactly with the change of scale in  $p$  observed in the case of the oxygen equilibrium. All the results developed for the oxygen equilibrium can, therefore, be applied to the case of the oxidation-reduction equilibrium subject to the identification given above. Thus in place of Equation 8 we have

$$(14) \quad \frac{\partial E_{01}}{\partial \text{pH}} = \frac{\partial E_{02}}{\partial \text{pH}} = \dots = \frac{\partial E_{04}}{\partial \text{pH}} = \left( \frac{\partial E}{\partial \text{pH}} \right)_z$$

and in place of Equation 9 we have

$$(15) \quad -\frac{F}{2.303RT} \left( \frac{\partial E}{\partial \text{pH}} \right)_z = B_1 - B_0 = \frac{B_2 - B_0}{2} = \frac{B_3 - B_0}{3} = \frac{B_4 - B_0}{4}$$

Here  $B_0, B_1 \dots B_4$  refer to the amounts of base bound per mole by Hb,  $\text{Hb}^+ \dots \text{Hb}^{++++}$  respectively. Taylor and Hastings' measurements were made at  $30^\circ$ . At this temperature the factor  $F/2.303RT = 16.63$ , and we see that  $16.63E$  replaces  $-\log 1/p$  in Equation 9. Corresponding to Equation 11 we have

$$(16) \quad -16.63E_z = \frac{1}{2} \log \frac{(k_1 + \text{H}) \dots (k_p + \text{H})}{(k'_1 + \text{H}) \dots (k'_p + \text{H})} + \text{constant}$$

In this equation the  $k$  values in the numerator are for  $\text{Hb}^{++++}$ , those in the denominator for  $\text{Hb}$ .  $E$ , it should be realized, is the voltage required to maintain a given percentage oxidation; i.e., a given value of  $Z$ .

There is just one more consideration. We can combine Equations 11 and 16 to give the following result.

$$(17) \quad 16.63E_Z = \log p_r + \frac{1}{4} \log \frac{(k_1 + H) \cdots (k_p + H)}{(k'_1 + H) \cdots (k'_p + H)} + \text{constant}$$

Here the  $k$  values in the numerator are for  $\text{Hb}(\text{O}_2)_4$ ; those in the denominator for  $\text{Hb}^{++++}$ .

The differential titration of  $\text{Hb}(\text{O}_2)_4$  and  $\text{Hb}^{++++}$  was carried out on horse hemoglobin prepared from red cells kindly furnished by the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health by the method described in earlier studies. In all, three preparations were made, one for each of the three experiments, Nos. 1, 2, and 3. In each case the hemoglobin was once recrystallized. Both the original crystals and the recrystallized material were washed carefully during the course of the preparation in order to remove impurities. It should be remarked, however, that in so far as impurities have the same acid- and base-combining power in the two solutions being compared, the results of the differential titration are unaffected by them.

A stock solution was made up for each experiment by dissolving the crystalline hemoglobin paste in 0.5 M NaCl to give as concentrated a solution as possible. All undissolved protein was removed by centrifuging. The stock solutions used for Experiments 1, 2, and 3 were calculated to contain  $1.59 \times 10^{-3}$ ,  $1.863 \times 10^{-3}$ , and  $1.858 \times 10^{-3}$  mole of hemoglobin and 0.400, 0.345, and 0.308 mole of NaCl per liter respectively. The figures for the hemoglobin concentrations are based on nitrogen analyses by the Kjeldahl method, with the assumption that 1 gm. of nitrogen corresponds to 5.93 (11) gm. of hemoglobin and that the molecular weight of hemoglobin is 66,800. The figures for the NaCl concentration are based on the observed increase of volume accompanying the solution of the hemoglobin paste.

In each experiment the solution of ferrihemoglobin used for titration was made up from a portion of the stock solution by addition of potassium ferricyanide. The corresponding solution

of  $\text{Hb}(\text{O}_2)_4$  for comparison was made by the addition of potassium chloride to another portion of the stock solution in an amount calculated to be equivalent to the ferricyanide, account being taken of the reduction of ferricyanide by the hemoglobin, in terms of ionic strength. To 10 cc. aliquots of these were added measured volumes of approximately 0.1 N HCl or NaOH and an additional amount of distilled water to make the final volume of each aliquot the same, the purpose being to have the ionic strength the same as far as possible for all titrated aliquots. The titrated aliquots were then placed in a glass electrode at 25° and the pH measured by means of a vacuum tube circuit as in earlier studies (7). The glass electrode was calibrated with standard phosphate buffers, on the basis of the pH values given by Clark (2). This means taking the pH of an equimolar mixture of M/15 mono- and dihydrogen phosphate as 6.80 at 25°. The slope of the calibration curve giving voltage as a function of pH agreed with the theoretical value within the limit of experimental errors. By neglecting any contributions to ionic strength arising from the hemoglobin itself, the solutions of ferrihemoglobin and oxyhemoglobin were calculated to have the following compositions when measured,  $\mu$  denoting ionic strength.

Experiment 1, a.	$\text{Hb}(\text{O}_2)_4$	$4.08 \times 10^{-3}$	hemes per liter,	$\mu = 0.325$
" 1, a.	$\text{Hb}^+$	$4.08 \times 10^{-3}$	" " "	" = 0.328
" 1, b.	$\text{Hb}(\text{O}_2)_4$	$3.15 \times 10^{-3}$	" " "	" = 0.242
" 1, b.	$\text{Hb}^+$	$3.15 \times 10^{-3}$	" " "	" = 0.246
" 2.	$\text{Hb}(\text{O}_2)_4$	$4.02 \times 10^{-3}$	" " "	" = 0.227
" 2.	$\text{Hb}^+$	$4.02 \times 10^{-3}$	" " "	" = 0.229
" 3.	$\text{Hb}(\text{O}_2)_4$	$4.04 \times 10^{-3}$	" " "	" = 0.209
" 3.	$\text{Hb}^+$	$4.04 \times 10^{-3}$	" " "	" = 0.208

In Experiment 1 the number of moles of ferricyanide added was nearly twice the number of hemes present in solution; in Experiment 3 it was exactly the same; and in Experiment 2 it was only 97 per cent of it, so that about 3 per cent of the hemoglobin may have been in the ferrous form  $\text{Hb}(\text{O}_2)_4$ . However, any corrections for Experiment 2 that might be based on this small figure would amount to less than the experimental error and none has been attempted. The errors in these experiments appear to be somewhat greater than those involved in the differential titration of Hb and  $\text{Hb}(\text{O}_2)_4$ , where the treatment of the two solutions is the

same until just before the measurement, when one is freed of oxygen by successive evacuations and equilibrations with nitrogen and the other is simply equilibrated with oxygen.

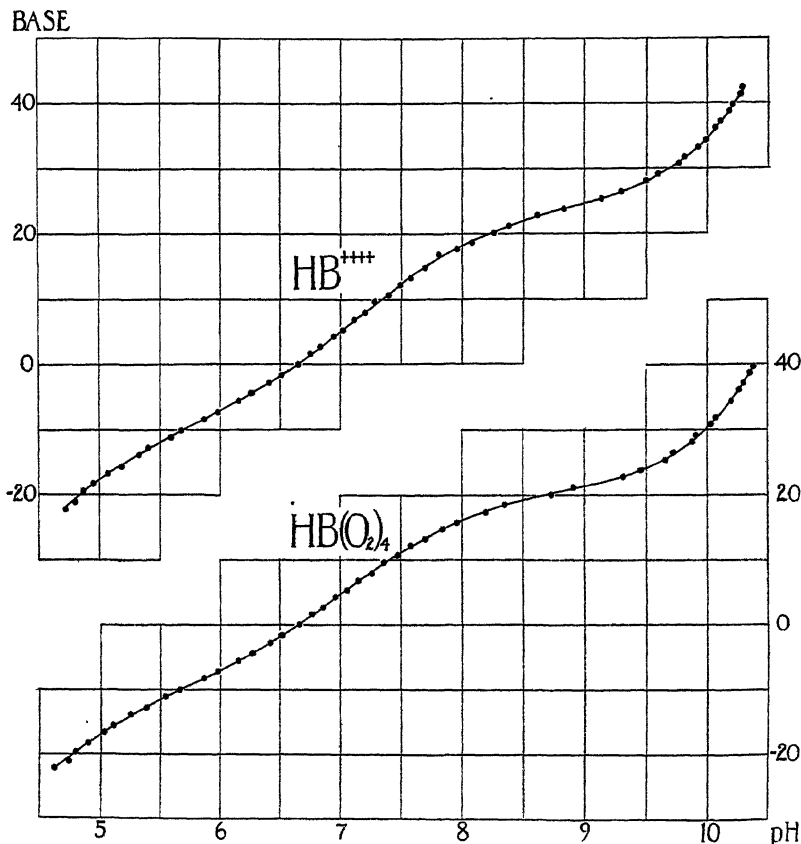


FIG. 1. Titration data of Experiment 3. Ordinates give equivalents of base added per mole (66,800 gm.) of hemoglobin.

The actual titration data of Experiment 3 are shown graphically in Fig. 1, which gives an idea of the consistency of the experimental results. What we are primarily interested in, however, is not the titration curves themselves, but rather the difference between them; that is, the difference in base bound per mole of ferrihemo-

globin and of oxyhemoglobin. It is this difference which is to be correlated with the rest of the experimental data. Values for this difference, read off from the smooth curves of Fig. 1 and from the corresponding graph (not shown) for Experiment 2 are given in Fig. 2. Experiment 1 was a preliminary one, and the results are less reliable than those of the other two experiments. For this reason they are not included in Fig. 2, although, except at pH values above 8.5, they are in very close agreement with the results of Experiments 2 and 3. Alkaline to this they indicate a noticeably greater difference in base bound between the two forms of hemoglobin.

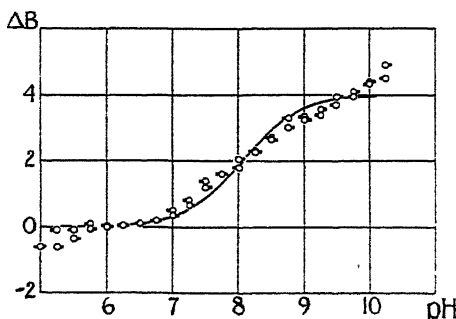


FIG. 2. Difference in the amount of base bound per mole (66,800 gm.) between  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$ . The circles tagged to the left and right represent Experiments 2 and 3, respectively.

All the previous experimental results on hemoglobin and oxyhemoglobin indicate that the four hemes are indistinguishable from one another, being related in the same way to identical portions of the globin. In fact the situation is the same as if the hemoglobin molecule were composed of four identical quadrants, one for each heme (13). Moreover it appears that each heme interacts with two acid groups, one of which is strengthened, the other of which is weakened, as a result of introducing oxygen into the heme (13). There is evidence, based in part on the heat of dissociation (12), in part on the heat of oxygenation in its relation to pH (13), that these two groups are imidazolium groups of histidine. We should expect therefore that if the transition from  $\text{Hb}(\text{O}_2)_4$  to  $\text{Hb}^{++++}$ , in which each of the four hemes is oxidized, produces any change at all in the acid properties of hemoglobin, this change

should consist of four identical effects on acid dissociation constants, one for each heme. Now the data shown in Fig. 2 can be reasonably well explained by assuming that oxidation leads to the appearance of four new identical acid groups, one for each heme, characterized by a  $pK$  value of 8.05 and that this is the *only* effect on the acid properties of the molecule produced by oxidation. The imidazolium groups appear not to be affected. The smooth curve of Fig. 2 is constructed on the assumption that the appearance of these four new acid groups is the only change in regard to acid properties produced by oxidation.

The idea that ferrihemoglobin has four identical acid groups, one for each heme, active in this range, is not new. The presence of four such groups each with a  $pK$  close to the value given above was inferred some years ago by Austin and Drabkin (1) as a result of spectroscopic studies, and had been suggested even earlier by Haurowitz (8). For canine hemoglobin at  $25^\circ \pm 1.5^\circ$  and at an ionic strength of 0.10, Austin and Drabkin give a value of 8.12 for  $pK$ , and point out that  $pK$  varies with ionic strength ( $\mu$ ) in accordance with the relation

$$(18) \quad pK = pK_0 + 0.6\sqrt{\mu}$$

Their conclusions are consistent with the results of Coryell, Stitt, and Pauling on bovine hemoglobin (5). On the basis of studies of magnetic susceptibilities these investigators show the presence in bovine ferrihemoglobin of four identical acid groups whose dissociation constant at  $24^\circ \pm 2^\circ$  is given by  $pK = 7.89 + 0.59\sqrt{\mu}$ . If we replace the factor 0.6 by 0.59, Austin and Drabkin's results on canine hemoglobin are equivalent to the relation

$$(19) \quad pK = 7.93 + 0.59\sqrt{\mu}$$

Our results on horse hemoglobin shown in Fig. 2 are for an ionic strength of about 0.22. Fitted to the same equation they correspond to the relation

$$(20) \quad pK = 7.77 + 0.59\sqrt{\mu}$$

In view of species difference the discrepancies are not unreasonable.

The novel, and for the present purpose, important thing about our results is that they represent a complete differential titration of  $Hb^{++++}$  and  $Hb(O_2)_4$  in the pH range covered. In distinction to this the spectroscopic and magnetic studies serve only to reveal



the presence of special groups whose dissociation leads to magnetic and spectroscopic changes; they yield accurate data regarding these groups, but they cannot give us the information we require concerning the *total* difference in acid properties between  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$ . Only an acid titration can do this. The results of the present differential titration, consistent as they are with those obtained by the other methods, provide the added information which could not have otherwise been foreseen, that between pH 5 and 10 there is no certain or considerable difference in acid properties between  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$  other than that associated with the appearance of the four groups also revealed by spectroscopic and magnetic observations.

This conclusion may now be applied to combine the results of Ferry and Green and those of Taylor and Hastings; in other words, to convert the values of  $E$  required to produce a given percentage oxidation into values of  $p$  required to produce a given percentage oxygenation, or conversely. The basis of this is provided by Equation 17. There remains only one obstacle. Ferry and Green's measurements were made at  $25^\circ$ , Taylor and Hastings' measurements at  $30^\circ$ . It is an easy matter, however, to convert Ferry and Green's results to  $30^\circ$ , since we are only interested in relative values of the oxygen pressure. It will be recalled that

$$\frac{\partial \log 1/p}{\partial \text{pH}} = \frac{(B_4 - B_0)}{4}$$

At the same time it has been observed (13) that the only effect on  $(B_4 - B_0)$  of changing the temperature is to change by a constant amount the pH values at which given values of  $(B_4 - B_0)$  occur. In terms of a graph in which  $(B_4 - B_0)$  is plotted against pH, this means that the effect of changing the temperature is to shift the curve parallel to itself along the pH axis. The magnitude of the shift is  $-0.016$  pH unit for each degree rise of temperature (13). Consequently it follows from Equation 20 for the  $5^\circ$  change from  $25^\circ$  to  $30^\circ$  that the curve for the relative values of  $1/p$  will be shifted 0.08 unit towards the acid end of the pH scale.

We have reconsidered the results of Ferry and Green and by fitting them with Pauling's equation (9) have reestimated the value of  $p$  corresponding to half saturation with oxygen for each pH studied by them. The basis for this was as follows: The

dissociation curves giving  $Y$ , the percentage saturation with oxygen, against  $p$ , the partial pressure of oxygen, at different pH values differ only by a scale factor involving  $p$ , and subject to this scale factor all conform to Pauling's equation (9). This simply means that the curves giving  $Y$  as a function of  $\log p$  differ only by a parallel shift along the pH axis and all conform within the limits of error to Pauling's equation plotted in this way. We therefore replotted Ferry and Green's data at each pH value in this manner and superposed them to the best advantage on the standard Pauling curve with the aid of a tracing table. This enabled us to estimate accurately and quickly the value of  $p$  cor-

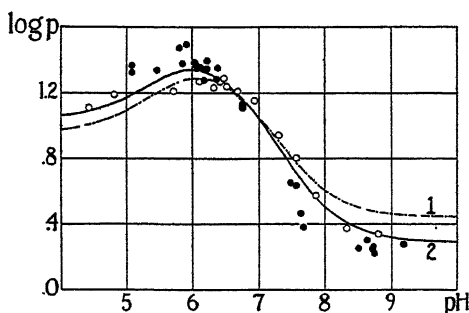


FIG. 3. O, values of  $\log p$ , the oxygen dissociation pressure at 50 per cent saturation, in relation to pH, from the data of Ferry and Green; ●, values of  $\log p$  calculated from the oxidation-reduction potentials given by Taylor and Hastings. Curves 1 and 2 are calculated on the basis of dissociation constants discussed in the text.

responding to half saturation. The data so obtained are shown in Fig. 3, the pH values being corrected to 30° by subtraction of 0.08 unit from each of the values recorded by Ferry and Green in accordance with the considerations of the last paragraph.

Fig. 3 also shows the  $\log p$  values calculated by Equation 17 from the oxidation-reduction voltages corresponding to half oxidation given by Taylor and Hastings. In making this calculation we have assumed as stated above that the only difference in regard to acid properties between  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$  is that due to the appearance of the four new identical pK values in  $\text{Hb}^{++++}$ . These have no observable counterparts in  $\text{Hb}(\text{O}_2)_4$ ; consequently we set the corresponding pK for  $\text{Hb}(\text{O}_2)_4 = \infty$  ( $K = 0$ ).

There is a question as to the exact value for these four new identical pK values in Hb<sup>++++</sup>. Taylor and Hastings' measurements were made at 30° and an average ionic strength of about 0.16. Equation 20 summarizes our observations at 25°. To convert this to 30° demands an assumption about the heat of dissociation of the new groups. It seems likely that in reality the dissociation represents the loss of a proton from a molecule of water coordinated with each ferriheme. We therefore take the heat of dissociation as 13,000 calories. This somewhat arbitrary assumption leads to the expression for pK at 30°

$$\text{pK} = 7.61 + 0.59 \sqrt{\mu}$$

which gives a value of pK at  $\mu = 0.16$  of 7.85, corresponding to a value of  $1.413 \times 10^{-8}$  for  $K$  itself. If we use this value, then Equation 17 gives

$$(21) \quad \log p = 16.63E + \log \frac{1.413 \times 10^{-8} + H}{H} + \text{constant}$$

It is the values of  $\log p$  calculated from this expression that are given in Fig. 3. In locating the values of  $\log p$  obtained in this way on the graph of Ferry and Green's data we are at liberty to raise or lower the points as a whole to secure the best agreement, owing to the presence of the constant of integration. This was conveniently done in preparing Fig. 3 by first plotting the calculated points on a separate sheet and then transferring them by means of a tracing table to the graph of Ferry and Green's data.

Fig. 4 gives the result of the converse procedure. The voltages given by Taylor and Hastings for half oxidation multiplied by the factor 16.63 and the values of  $16.63E$  calculated by Equation 21 are shown. In both cases the agreement is fairly good, although by no means perfect. The discrepancies seem to be larger than the accuracy of either set of measurements, as judged by the ability to determine either  $\log p$  or  $E$  or by the consistency of the results.

It would, of course, be possible to improve the agreement by postulating *ad hoc* additional pK differences between Hb<sup>++++</sup> and Hb(O<sub>2</sub>)<sub>4</sub>. The magnitude of the corresponding pK values may be obtained by taking the differences of ordinate between smooth curves (not shown) drawn through the open and solid

circles in Fig. 3 and plotting them against pH. The curve so obtained should be capable of being fitted by an expression

$$\log \left( \frac{k_1 + H}{k'_1 + H} \right) \left( \frac{k_2 + H}{k'_2 + H} \right) \cdots + \text{constant}$$

If we try this, limiting ourselves to a single pK shift for each heme, the required pK values turn out to be about 6.38 for  $\text{Hb}^{++++}$  and

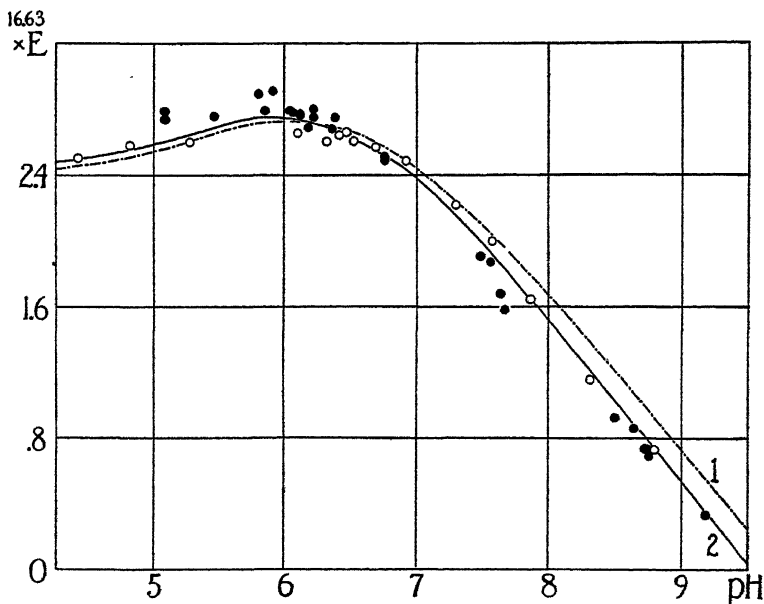


FIG. 4. ●, oxidation-reduction voltages  $\times 16.63$  for 50 per cent oxidation, from the data of Taylor and Hastings; ○, values of the same calculated from the oxygen dissociation pressures given by Ferry and Green. Curves 1 and 2 are calculated on the basis of dissociation constants discussed in the text.

6.72 for  $\text{Hb}(\text{O}_2)_4$ . If we accepted this analysis, we should be constrained to conclude that besides the introduction of the four new constants ( $\text{pK} = 7.85$ ) oxidation also produces an effect on one additional group for each heme; namely, a group whose pK is roughly 6.72 in  $\text{Hb}(\text{O}_2)_4$  at  $30^\circ$ . This group would naturally be identified with the imidazolium group whose pK has been previously estimated as 6.80 at  $25^\circ$  in  $\text{Hb}(\text{O}_2)_4$  (13). Such a con-

clusion would not in itself be unreasonable, but it fails to accord with the titration experiments. It would imply a differential titration curve for  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$  quite different from the smooth curve in Fig. 2, and one which agrees less well with the experimental points. It would demand, for example, a difference of about 0.56 equivalent in the amount of base bound between  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$ . Every single titration carried out indicated a difference of 0.1 equivalent or less at this pH. It seems better, therefore, to ascribe the systematic discrepancies in Figs. 3 and 4 to variations in activity coefficients of the protein molecules with pH.

It is possible, as we showed in an earlier study (13), to explain the observed differential titration of  $\text{Hb}(\text{O}_2)_4$  and Hb quite exactly in terms of two pK shifts per heme; namely, one shift from 5.25 to 5.75 and another shift from 7.81 to 6.80 accompanying the change from Hb to  $\text{Hb}(\text{O}_2)_4$ . These shifts occur in groups whose heat of dissociation is 6500 calories, presumably imidazolium groups of histidine (13). The above figures are for 25°. Reduced to 30° (on the basis of 6500 calories) they become 5.17, 5.67, 7.73, and 6.72. By introducing these constants into Equation 11 we obtain for 30°

$$(22) \quad \log p = \log \frac{(6.761 \times 10^{-8} + \text{H}) (1.862 \times 10^{-8} + \text{H})}{(2.138 \times 10^{-8} + \text{H}) (1.905 \times 10^{-7} + \text{H})} + \text{constant}$$

Curve 1 in Fig. 3 corresponds to this equation. Agreement with the experimental points is not very good. Trial and error show that a much better fit of the Ferry and Green data can be obtained with a somewhat different choice of pK values, although any such change reduces the fit of the differential titration data of German and Wyman for  $\text{Hb}(\text{O}_2)_4$  and Hb. About the best fit of the oxygen data is obtained if we leave the constants 5.17 and 5.67 unaltered but change the constants 6.72 and 7.73 to 6.60 and 7.85. Curve 2 in Fig. 3 is calculated from the latter values. The extent to which this alternative choice of constants satisfies the differential titration data of German and Wyman is seen in Fig. 5. The curve is based on the modified constants reduced to 25°; viz., 5.25, 5.75, 6.68, and 7.93. The extent of the disagreement between the curve and the observations appears to be greater than the experi-

mental error, but is by no means sufficient to rule out the change. It is difficult to deoxygenate hemoglobin completely, particularly in the alkaline range of pH where the oxygen affinity is high, and the discrepancy, which is greatest here, might be explained by supposing that the hemoglobin was never completely freed of oxygen in the course of the differential titrations. The measured difference in base bound between two solutions at the same pH, one of oxygenated hemoglobin and the other of partially deoxygenated hemoglobin, should be proportional to the percentage deoxygenation. If we accept the new choice of constants, Fig. 5

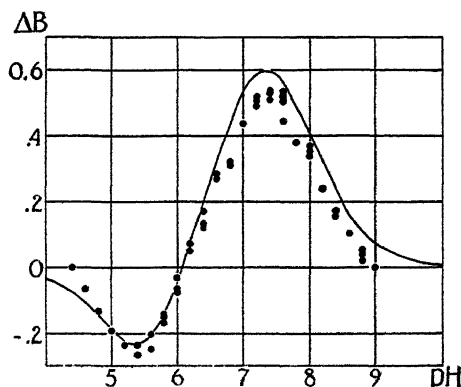


FIG. 5. Differential titration data for  $\text{Hb}(\text{O}_2)_4$  and Hb, from German and Wyman. The ordinates represent equivalents of base per heme (16,700 gm.). The curve is calculated on the basis of assumptions discussed in the text.

shows that in our differential titrations we must have been dealing, in the region of pH 7 or 7.5, with hemoglobin which was only about 85 or 90 per cent deoxygenated. The only other course would be to stick to the original constants (5.17, 5.67, 7.73, and 6.72) postulated to account for the differential titration and ascribe the whole discrepancy to the change of activity coefficients with pH.

It is, of course, also possible to calculate the values of  $E$  from the pK differences between  $\text{Hb}^{++++}$  and Hb, and these differences are fixed by the differential titration once we accept the pK differences between  $\text{Hb}(\text{O}_2)_4$  and Hb. If we accept for the latter the

original values (5.17, 5.67, 7.73, and 6.72) and ascribe the differential titration of  $\text{Hb}^{++++}$  and  $\text{Hb}(\text{O}_2)_4$  wholly to the appearance of four new constants,  $\text{pK} = 7.85$ , then we have for the change from Hb to  $\text{Hb}^{++++}$  at  $30^\circ$  the following pK shifts per heme:  $5.17 \rightarrow 5.67$ ,  $7.73 \rightarrow 6.72$ ,  $\infty \rightarrow 7.85$ . Values of  $16.63E$  calculated on the basis of these constants from Equation 16 correspond to Curve 1 in Fig. 4. If instead of these original constants for Hb and  $\text{Hb}(\text{O}_2)_4$  we use the alternative values which give a better fit to the oxygen values, then we obtain for the pK shifts per heme accompanying the change from Hb to  $\text{Hb}^{++++}$  at  $30^\circ$  the following figures:  $5.17 \rightarrow 5.67$ ,  $7.85 \rightarrow 6.60$ ,  $\infty \rightarrow 7.85$ . There is a cancelation of the effects of the two constants 7.85, one for  $\text{Hb}^{++++}$ , one for Hb, of the type suggested as a possibility by Coryell and Pauling (3). The net effect is equivalent to the following shift:  $5.17 \rightarrow 5.67$ ,  $\infty \rightarrow 6.60$ . Curve 2 in Fig. 4 is based on these constants and agrees with the observations fairly well. Disagreement is certainly small enough to be explicable in terms of activity coefficient changes. It might be pointed out that in the absence of all other data an almost perfect fit of the results of Taylor and Hastings could be achieved by an *ad hoc* choice of the following pK shifts:  $5.17 \rightarrow 5.67$ ,  $\infty \rightarrow 6.40$ ; but such a choice would be artificial.

In conclusion it appears that the best way to satisfy all four bodies of experimental material, namely the two differential titrations, the results of Ferry and Green, and those of Taylor and Hastings, is to assume the following pK values per heme for  $30^\circ$  and  $\mu = 0.16$ .

Hb	$\text{Hb}(\text{O}_2)_4$	$\text{Hb}^{++++}$
5.17	5.67	5.67
7.85	6.60	6.60
$\infty$	$\infty$	7.85

At  $25^\circ$  and  $\mu = 0.16$  these values become

Hb	$\text{Hb}(\text{O}_2)_4$	$\text{Hb}^{++++}$
5.25	5.75	5.75
7.93	6.68	6.68
$\infty$	$\infty$	8.01

Nothing has been said as to the variations of the constants given in the first two lines of this tabulation with ionic strength. Presumably it should be about the same as that of the constant 7.93

whose variation with ionic strength has been given by Austin and Drabkin and by Coryell, Stitt, and Pauling. All discrepancies between the data which persist after the acceptance of these constants are certainly small enough to be reasonably attributed to experimental errors, to variations in activity coefficients, or both.

#### SUMMARY

This paper presents the results of a differential acid-base titration of oxygenated hemoglobin against ferrihemoglobin. General relations are developed which make it possible to use these data to calculate values of the oxygen dissociation pressures from the oxidation-reduction voltages, and conversely. Such calculations are made on the basis of the results of Ferry and Green and of Taylor and Hastings. The results show that the three sets of data are reasonably consistent: the relatively small discrepancies in excess of experimental errors may be attributed to variations of activity coefficients with pH. An analysis of all the data is given in terms of dissociation constants of hemoglobin, ferrihemoglobin, and oxyhemoglobin.

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## LIPIDS OF THE FASTING MOUSE

### I. THE RELATION BETWEEN CARCASS LIPIDS AND LIVER LIPIDS\*

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#### PLATE 6

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Fasting as a tool in the study of fat metabolism has a unique usefulness. Fat is a food; stored fat is to be reckoned as stored fuel. Subjecting an animal to such conditions that it must exist on its own tissues will show the availability of such fat reserves and something of the manner in which they are mobilized, transported, and disposed of. Changes in the amount of carcass fat will reveal the mobilization rate; changes in the character of the carcass fats will reflect the mechanism of the withdrawal of fat from the depots. The nature and amount of the irreducible minimum of carcass fat will indicate what fatty substances have an essential and presumably a structural rôle in carcass tissues. The liver has long been indicated to be the primary destination of mobilized body fat. Chemical changes in the various lipid fractions of this organ will provide a basis for understanding not only its rôle in metabolizing fat but also the importance of various lipids in the function and structure of the liver. Consequently, studies of the simultaneous changes in carcass and liver fats in fasting animals should be enlightening both as to fat metabolic processes and as to the structural functions of lipids.

Fasting as a tool in the study of fat metabolism is a time-honored

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procedure. A number of investigators have reported chemical studies of the liver fats during fasting (*e.g.*, Mottram, Dible, Junkersdorf, Rothschild (1-4)) which have clearly indicated this organ's vital rôle in the combustion of fats from the depots. The tenacity with which the liver holds onto a certain minimal amount of fat has also been observed. This has been taken to indicate that the liver needs fat for its structure and function.

Chemical studies of the fat metabolism in the fasting white mouse have been limited and relatively few in number (5-9). In each case, the marked increase in total liver lipids has been a point of major interest. Various factors, such as age of the mouse, body weight, previous diet, room temperature, and water deprivation, have been shown to cause wide variations in the lipid changes. However, in no case was a differential analysis of the liver lipids carried out along with a determination of the body changes. As a matter of fact, the literature does not contain an adequate statement as to the normal lipid distribution of mouse liver (10, 11). Because the mouse has a comparatively rapid fat metabolism (6) and because micromethods now make differential lipid analyses feasible on a single mouse liver, the data reported below direct attention to a field deserving further investigation.

Histological studies of changes in mouse liver during fasting are numerous (bibliography of (12)); correlated chemical changes have not been investigated. The present study offered an excellent opportunity to compare estimations of sudanophil lipid content with the chemical analyses.

### *Procedure*

3 month-old, male, albino mice were used which, for the most part, had been reared in our laboratory on a diet of oats and Purina Dog Chow. After initial weighing, the mice were fasted (water *ad libitum*) in individual cages which had coarse screens for flooring, so that coprophagy was minimal. The temperature of the room was reasonably constant, but varied from 20-25°. The mice were sacrificed by a blow on the head. The livers were removed as quickly as possible; a certain lobe was taken routinely, weighed, and placed in a fixative for histological examination. The remainder of the liver was weighed and placed in 95 per cent ethyl alcohol. It was then ground with sand, extracted, and

analyzed according to standard lipid methods (13-15). The carcasses were placed in 10 per cent sodium hydroxide solution, warmed until dissolution was complete, acidified, ether-extracted, and analyzed. The total fatty acids were determined gravimetrically, the iodine numbers by the method of Yasuda (16), and the total cholesterol was determined on the total lipid extract by the Liebermann-Burchard reaction.

*Histochemical*—A certain lobe of each liver was fixed for at least 2 days in 10 per cent formol-saline, prepared by dissolving 8.5 gm. of sodium chloride in 100 cc. of commercial formalin and diluting the solution to 1 liter with distilled water. Sections were cut at 25  $\mu$  on a freezing microtome and (a) stained for lipid with Sudan IV, no counterstain being used, and (b) treated by the Schultz method for cholesterol (17).

### Results

*Mortality Data*—The mortality increased rapidly with the number of days of fasting; viz., 0, 13, 39, and 66 per cent for the four fasted groups, respectively. The loss by the 4th day was sufficient to make the study of mice fasted 5 or more days impractical. Although Best and Campbell (7) do not mention the age of their mice, presumably old mice were used, since they report data up to 7 days of fasting.

*Initial Body Weight*—The average initial body weights for the five groups of mice ranged from 22 to 24 gm. (Tables I to V).<sup>1</sup> No serious distortions of the average analytical data (as a result of the variation in body weight) were found except from including the data for the three mice weighing 27 gm., Mice 85, 88, and 104, fasted 4 days, in which the liver lipid values were excessively high. The constancy of the initial body weights implies a uniformity of stored fat at the beginning of fasting.

*Carcass Weight*—The carcass weights plus the liver weights were taken as the body weights at the time of sacrifice; the weight of a few drops of blood lost from certain mice was neglected.

<sup>1</sup> By oversight, the mice in the groups fasted 1 day and 4 days were not weighed initially; for these mice initial body weights were calculated from the carcass weights and the average weight loss curve, based on 67 mice observed for 1 day of fasting, 59 for 2 days, 25 for 3 days, and 19 for 4 days.

*Total Lipid Content of Carcass*—The total carcass lipid of the normal mice was about 9 per cent of the body weight. 1 day's fasting lowered the lipid to 5 per cent and 2 days to 2 per cent,

TABLE I  
*Data on Carcass and Liver Lipids of Normal Mice*

Mouse No.	Initial body weight	Carcass					Liver						
		Weight of body without liver	Total lipid	Total fatty acid I No.	Total cholesterol	Total weight	Sample weight	Total lipid	Sudanophil lipid†	Phospholipid	Cholesterol		
											Total	Ester	
	gm.	gm.	gm.		mg.	gm.	gm.	gm.		mg.	mg.	per cent	
25	20	18.41	1.205	92	5.5	1.363	1.191	69	M.	46	3.1	43	
26	21	20.01	1.318	90	5.8	1.391	1.223	74	"	48	4.4	19	
27	21	19.38	1.186	92	6.5	1.393	1.079	66	S.	46	2.7	15	
28	22	20.73	3.200	86	6.0	1.549	1.404	99	M.	58	3.5	27	
29	22	20.96	1.432	90	6.5	1.484	1.320	85	"	53	4.4	10	
30	23	21.94	1.843	87	6.4	1.402	1.266	80	"	51	3.5	23	
31	22	20.32	0.947	97	6.2	1.051	0.842	62	"	42	2.5	24	
32	23	21.95	1.093	94	6.0	1.340	1.036	72	"	43	3.1	24	
33	21	19.43	1.615	83	6.0	1.227	1.022	70	"	43	4.3	14	
34	18	16.67	1.000	86	5.3	1.041	0.783	55	"	39	5.2	11	
35	23	22.03	2.229	83	6.8	1.276	1.157	64	S.	49	5.2	16	
36	23	21.71	2.473	84	6.4	1.522	1.325	73	"	54	5.3	10	
37	25	23.60	2.863	86	6.5	1.439	1.274	78	M.	56	5.8	16	
38	25	23.30	2.830	83	7.1	1.524	1.350	92	"	54	6.3	19	
39	24	22.98	2.079	82	7.1	1.381	1.188	77	"	54	8.0	14	
40	22	20.25	1.839	85	6.1	1.450	1.312	81	"	54	5.3	18	
41	22	20.49	1.734	82	5.6	1.302	1.126	77	"	49	5.3	14	
42	18	17.33	1.761	82	5.0	0.912	0.792	55	"	35	4.0	21	
43	22	20.89	1.940	81	5.9	1.176	1.031	72	"	47	6.7	21	
44	26	24.57	1.658	82	7.0	1.426	1.291	72	S.	54	3.6	35	
Average	22	20.8	1.81	87	6.2	1.332	1.151	74		49	4.6	20	
σ.....		2	0.7	5	0.6			10		6	1.2	7	

\* M. = moderate; S. = scanty.

where it remained. Apparently 2 per cent of the body weight is essential lipid and the other 6 to 8 per cent is depot fat. The body fat loss is a constant percentage (40 per cent) of the body weight loss during the first 2 days.

Barrett, Best, and Ridout (9) in a somewhat similar experiment found somewhat higher values for total depot fat per mouse (2.6

TABLE II  
*Data on Carcass and Liver Lipids of Mice Fasted 24 Hours*

Mouse No.	Initial body weight	Carcass					Liver					
		Weight of body without liver	Total lipid	Total fatty acid I No.	Total cholesterol	Total weight	Sample weight	Total lipid	Sudanophil lipid*	Phospholipid	Cholesterol	
											Total	Ester
	gm.	gm.	gm.		mg.	gm.	gm.	mg.		mg.	mg.	per cent
45	23	21.17	1.111	89	5.8	1.142	1.009	80	M.	40	5.2	22
46	22	20.46	0.898	88	5.3	1.176	1.062	74	"	39	3.2	35
47	21	19.26	1.277	83	5.7	1.447	1.273	212	A.	45	9.0	19
48	23	21.05	1.156	87	6.2	1.077	0.959	141	"	40	5.3	35
49	20	18.19	1.006	86	5.7	0.934	0.802	140	"	32	4.7	49
50	20	18.85	1.013	92	5.9	1.209	1.056	200	"	40	6.0	26
51	23	21.18	0.963	92	6.6	1.156	0.987	88	"	44	5.1	23
52	20	18.70	0.788	94	6.1	1.380	1.261	153	"	44	5.4	29
53	21	19.64	1.535	88	5.6	1.327	1.142	130	"	44	6.1	23
54	22	20.81	1.152	93	5.9	1.131	0.952	117	"	42	4.8	48
55	24	23.16	1.756	90	5.9	1.101	0.980	129	"	39	5.3	21
56	22	21.25	1.018	93	6.6	1.092	0.960	159	"	34	5.5	27
57	24	22.63	1.427	89	6.1	1.150	1.004	186	"	38	5.5	42
58	21	19.89	0.352	110	5.7	0.978	0.848	38	S.	29	3.9	17
59	21	20.19	0.825	96	6.5	1.145	0.976	110	A.	40	5.2	19
60	23	21.58	1.334	91	6.5	1.295	1.105	128	"	39	5.3	53
61	20	19.00	0.449	105	6.4	0.819	0.698	37	S.	28	3.4	13
62	24	22.85	2.236	85	6.3	1.240	1.076	192	A.	37	5.2	13
63	21	19.51	0.914	91	6.3	1.188	1.040	121	"	40	5.2	30
64	21	19.41	1.502	87	5.4	1.175	1.036	167	"	34	4.7	26
Average	22	20.4	1.14	92	6.0	1.158	1.011	130		38	5.2	28
σ..		1.4	0.4	6	0.4			49		5	1.0	13

\* A. = abundant; M. = moderate; S. = scanty.

to 6.2 gm.) than are given in Table I ( $1.8 \pm 0.7$  gm.). This difference would be accounted for if they used older mice.

*Linear Relation of Logarithm of Total Lipid of Carcass to Total Fatty Acid Iodine Number*—Fasting produces an increase in the

average iodine number of the remaining fatty acids (Table VI). In an attempt to discover a simple relation between total body fat and iodine number, the entire 100 mice were regrouped on the

TABLE III  
*Data on Carcass and Liver Lipids of Mice Fasted 2 Days*

Mouse No.	Initial body weight	Carcass				Liver						
		Weight of body without liver	Total lipid	Total fatty acid I No.	Total cholesterol	Total weight	Sample weight	Total lipid	Sudanophil lipid*	Phospholipid	Cholesterol	
											Total	Ester
	gm.	gm.	gm.		mg.	gm.	gm.	mg.		mg.	mg.	per cent
105	22	17.48	0.314	100	5.8	0.561	0.496	23	S.†	17	6.0	31
106	21	17.10	0.762	89	5.4	0.883	0.810	159	A.	29	7.6	25
107	24	17.36	0.320	103	5.1	0.718	0.622	32	S.	25	5.7	19
108	22	17.64	0.697	89	5.4	1.078	0.964	204	A.	34	6.8	17
109	20	15.50	0.342	101	5.0	0.632	0.616	29	M.†	21	5.2	4
110	23	18.09	0.434	99	5.8	0.855	0.777	58	"	29	5.6	39
111	21	17.38	0.386	97	5.2	0.849	0.785	48	"	30	4.3	22
112	22	17.95	0.380	102	5.8	0.781	0.642	35	"†	27	5.0	15
113	21	15.94	0.311	102	5.1	0.743	0.671	33	S.	25	4.8	10
114	22	17.11	0.281	109	4.5	0.494	0.434	23	"†	15	6.8	37
115	20	15.65	0.372	101	5.8	0.831	0.712	96	A.	29	6.2	17
116	21	15.52	0.290	107	5.1	0.743	0.654	30	S.	23	5.5	12
117	22	16.13	0.346	101	5.5	0.667	0.584	29	M.†	23	3.8	22
118	22	16.56	0.309	108	5.4	0.579	0.514	28	S.	19	4.8	57
119	20	15.00	0.323	101	5.0	0.715	0.616	54	M.	28	5.8	42
120	23	17.37	0.350	90	5.0	0.789	0.677	40	"†	26	4.8	21
121	23	17.71	0.520	99	5.6	0.973	0.890	117	A.	34	6.0	55
122	23	18.50	0.481	99	5.6	0.939	0.835	131	"	32	5.9	54
123	20	15.44	0.302	108	5.1	0.626	0.517	27	S.	20	4.0	17
124	21	15.96	0.337	102	5.0	0.461	0.410	23	"	15	5.0	78
Average	22	16.8	0.39	100	5.3	0.746	0.662	61		25	5.5	30
σ...		1.0	0.1	6	0.3			51		6	2.8	25

\* A. = abundant; M. = moderate; S. = scanty.

† The Schultz reaction was doubtful; in all other livers, the Schultz lipid test was negative.

basis of the amount of carcass lipids. This classification showed that the iodine numbers increase slowly until about three-fourths of the utilizable lipids is burned and thereafter increase very rap-

idly. Plotting the average iodine numbers against the logarithms of the average total carcass lipids (Text-fig. 1) gives evidence of a

TABLE IV  
*Data on Carcass and Liver Lipids of Mice Fasted 3 Days*

Mouse No.	Initial body weight	Carcass					Liver						
		Weight of body without liver	Total lipid	Total fatty acid I No.	Total cholesterol	Total weight	Sample weight	Total lipid	Sudanophil lipid*	Phospholipid	Cholesterol		
											Total	Ester	
	gm.	gm.	gm.		mg.	gm.	gm.	mg.		mg.	mg.	per cent	
65	24	17.53	0.488	100	6.1	0.992	0.882	59	M.	33	3.3	21	
66	24	18.46				0.781	0.698	28		22	3.5	27	
67	26	17.28	0.329	104	5.3	0.654	0.545	26	S.	21	3.4	21	
68	24	17.47	0.284	111	5.1	0.551	0.491	21	"	15	4.6	33	
69	26	17.57	0.368	106	5.6	0.728	0.656	29	"	22	4.0	40	
70	26	17.89	0.356	107	5.8	1.002	0.889	25	"†	18	3.3	15	
71	24	16.70	0.281	109	5.0	0.561	0.510	22	"	17	3.8	12	
72	26	17.80	0.310	110	5.6	0.627	0.541	26	"	20	4.2	14	
73	24	15.29	0.333	108	5.9	0.537	0.472	23	"	16	5.0	21	
74	25	17.36	0.372	111	6.3	0.576	0.480	24	"	17	6.0	26	
75	25	17.83	0.765	92	5.6	0.990	0.883	60	A.	35	3.7	12	
95	20	17.16	0.316	113	5.0	0.575	0.483	23	S.	17	4.4	20	
96	24	18.60	0.675	97	6.1	0.833	0.720	61	M.	28	4.2	35	
97	24	17.62	0.380	105	5.8	0.596	0.520	26	S.†	18	4.6	46	
98	25	18.84	0.336	104	5.5	0.673	0.607	28		22	2.8	20	
99	23	16.67	0.328	102	5.3	0.594	0.529	24	S.	18	1.2	58	
100	25	19.14	0.757	98	6.6	0.968	0.879	71	M.	36	4.9	31	
101	24	18.49	0.344	108	5.8	0.732	0.642	31	S.†	23	4.4	18	
102	26	19.54	0.398	104	6.0	0.797	0.708	32	"	24	3.8	21	
103	23	18.60	0.316	117	6.0	0.517	0.442	20	"	14	3.3	19	
Average...	24	17.8	0.41	106	5.7	0.714	0.628	33		22	3.9	25	
σ. ....		1.0	0.1	6	0.4			15		6	1.0	13	

\* A. = abundant; M. = moderate; S. = scanty.

† The Schultz reaction was doubtful; in all other livers, the Schultz lipid test was negative.

linear relation. The equation of this line (calculated by the method of least squares) is as follows:

$$\text{Iodine number} = 92.2 - 23.1 \log \text{total lipid (gm.)} \quad (1)$$



In Text-fig. 1, the straight line is the graph of the equation; the average class values and the respective averages observed for the normal and fasted groups (Table VI) are also shown.

TABLE V  
*Data on Carcass and Liver Lipids of Mice Fasted 4 Days*

Mouse No.	Initial body weight	Carcass				Liver						
		Weight of body without liver	Total lipid	Total fatty acid I No.	Total cholesterol	Total weight	Sample weight	Total lipid	Sudanophil lipid*	Phospholipid	Cholesterol	
											Total	Ester
	gm.	gm.	gm.		mg.	gm.	gm.	mg.		mg.	mg.	per cent
76	21	14.81	0.252	100	6.6	0.763	0.682	30	S.	25	2.2	31
77	21	15.24	0.744	87	5.0	0.780	0.695	75	A.	29	3.2	26
78	23	16.36	0.236	93	6.7	0.647	0.551	26	S.	19	7.4	23
79	25	17.76	0.310	94	4.9	0.806	0.713	23	"†	16	4.8	28
80	20	13.42	0.261	103	6.6	0.518	0.466	20	"	15	2.6	28
81	22	15.50	0.657	92	5.3	0.834	0.734	57	M.	32	2.5	23
82	21	15.20	0.280	109	5.1	0.684	0.608	29	S.	24	2.3	34
83	24	18.43	0.419	101	6.4	0.921	0.806	48	M.	34	2.6	42
84	20	14.25	0.326	98	5.0	0.740	0.660	30	S.	23	3.3	13
85	27	20.04	1.238	93	7.1	0.975	0.883	98	A.	35	2.9	41
86	23	16.20	1.077	90	5.6	0.894	0.776	96	"	31	3.7	27
87	20	14.20	0.321	103	5.0	0.694	0.615	30	S.	24	2.2	24
88	27	21.57	1.866	88	6.8	0.959	0.835	103	A.	37	3.7	29
89	24	18.30	0.389	99	5.6	0.814	0.732	39	M.	26	3.5	46
90	22	15.96	0.374	98	5.4	0.572	0.499	23	S.	16	3.2	55
91	23	16.65	0.454	100	4.9	0.867	0.768	47	M.	31	2.9	22
92	23	16.57	0.323	107	5.0	0.634	0.542	31	S.	24	3.6	14
93	23	18.23	0.408	102	5.8	0.605	0.511	25	"	18	2.6	29
94	22	15.87	0.323	109	5.5	0.567	0.486	23	"	17	3.2	21
104	27	20.78	1.770	92	7.0	1.196	1.042	305	A.	40	4.7	100
Average..	23	16.8	0.60	98	5.7	0.773	0.680	58		26	3.3	33
σ . . . .		2.2	0.5	6	0.8			62		7	1.4	18

\* A. = abundant; M. = moderate; S. = scanty.

† The Schultz lipid test was positive.

With Equation 1, iodine numbers have been calculated for the average total lipids observed in the various fasted groups; these data are also given in Table VI and show a good agreement with

the observed iodine numbers. Interpreted broadly, the linear relation signifies that when a certain amount of carcass lipid is burned the iodine number increase is proportional to the amount of total lipid still available.

*Non-Selective Utilization of Carcass Lipids*—A simple calculation based on the amounts of carcass total lipid and the respective iodine numbers indicates that the changes observed may well arise from a non-specific burning of fat with an iodine number of approximately 80. For the group fasted 1 day, the calculated iodine number was 92; the average value observed was 90. For the

TABLE VI  
*Data on Carcass Lipid Changes during Fasting (Averages for Mice Weighing 20 to 24 Gm.)*

Duration of fasting	No. of mice	Total lipid				Total cholesterol		Total fatty acid I No.		
		Weight	Per cent change from normal	Per cent change body weight	Per cent change from normal	Weight	Per cent change from normal	Observed	Per cent change from normal	Calculated*
<i>days</i>		<i>gm.</i>				<i>mg.</i>				
Normal	15	1.96		8.6		63		86		85
1	20	1.13	-42	5.4	-37	60	-5	92	+7	91
2	20	0.39	-80	2.3	-73	53	-16	101	+17	102
3	14	0.42	-79	2.3	-73	57	-10	105	+23	101
4	17	0.42	-79	2.3†	-73	55	-13	100	+17	101

\* Calculated from Equation 1.

† Mouse 86 was omitted from the average.

groups fasted 2 days or longer, the calculated iodine number of the remaining lipid was 109 to 114; the average value observed was 101. However, from the primary data (Tables I to V), it may be seen that of the forty-seven mice having a terminal total carcass lipid of less than 0.5 gm., twenty-one had an iodine number greater than 104 and thirteen greater than 108, so that the calculated values are not absurd physiologically. There is no evidence of a preferential or selective utilization of the fatty acids. Longenecker (18) recently came to similar conclusions from fasting and refeeding studies.

*Liver Phospholipid to Neutral Fat Ratio*—It is particularly interesting to observe that the normal ratio of 67:26 is almost exactly reversed in the livers of the group fasted 1 day; *viz.*, 29:66. Since the phospholipid concentration is constant, this reversal is entirely due to an increase of neutral fat.

*Carcass and Liver Cholesterol*—On the 1st day 3 mg. of cholesterol are lost from the carcass; of this, 20 per cent appears as ester cholesterol in the liver. On the 2nd day 5 mg. are lost of which only 6 per cent appears as ester cholesterol in the liver. Thereafter no further cholesterol is lost from the carcass but 2 mg. of cholesterol disappear from the liver. Whether the disappearing cholesterol is excreted or burned cannot be stated, but the combustion of this amount of cholesterol<sup>2</sup> is within the demonstrated ability of the mouse (19).

The source of the cholesterol appearing in the liver may be tentatively taken to be the depot fat. No analyses are available on the cholesterol of mouse depot fat. However, 1 mg. of cholesterol per gm. of depot fat would be sufficient to account for the observed increase in the livers.<sup>3</sup> From Text-fig. 2, the total cholesterol percentages in the liver are seen to require 2 days of fasting to reach a maximum.

*Loss of Non-Lipid Constituents*—The daily non-lipid losses are 1.1, 1.3, 1.3, and 0.9 gm., which shows a remarkably constant metabolic demand on these body tissues.

*Histochemical*—The livers were classified according to whether the sudanophil lipid was abundant (Fig. 1), moderate (Fig. 2), or scanty (Fig. 3). Table VIII shows the numbers of livers in each class.

Table VIII shows that lipid was seen in all the normal livers and that, on the average, lipid was abnormally abundant after 1 day's fasting, abnormally scanty after 3 days, and, except for the three mice weighing 27 gm., abnormally scanty after 4 days fasting. By ranking the livers histologically as shown in Table VIII, the differences may be tested statistically. Thus, if the "abundant" grade is given a rank of 3, "moderate" of 2, and

<sup>2</sup> Schoenheimer and Breusch (19) reported that under suitable conditions a mouse can destroy 4 to 6 mg. of cholesterol per day.

<sup>3</sup> Schuette *et al.* (20) have reported 0.4 per cent unsaponifiable matter in horse depot fat.

"scanty" of 1, averages and *s* values for each day may be taken. From this analysis, significance may be safely attached to the differences between normal mice and each of the groups fasted 1 and 3 days and to the differences between the groups fasted 1 *versus* 2 days and 2 *versus* 3 days. The other differences either are not significant or have a probability of chance occurrence not exceeding ten chances in 100 trials (21).

The result of the Schultz cholesterol test was positive (lipid, blue-green) for only one liver from a mouse fasted 4 days. It was doubtful for six livers from mice fasted 2 days and for three from

TABLE VIII

*Neutral Fat Content of Livers Ranked Histologically According to Whether Sudanophil Lipid Was Abundant, Moderate, or Scanty*

Duration of fasting	Abundant		Moderate		Scanty	
	No. of mice	Neutral fat	No. of mice	Neutral fat	No. of mice	Neutral fat
<i>days</i>		<i>mg.</i>		<i>mg.</i>		<i>mg.</i>
Normal			16	22	4	13
1	16	103	2	32	2	5
2	5	105	7	10	8	2
3	1	21	3	10	16*	2
4	5†	67	4	14	11	3
Average.		99		19		3

\* Including two livers in which no lipid was seen.

† Three of these mice weighed 27 gm. initially and were found to be atypical in the total lipid content of their livers (Table V).

mice fasted 3 days. For all other livers, including the normal, the result was negative (lipid, brown).

*Histological and Chemical Correlations*—When the total liver lipid percentages were averaged for the groups of mice classified on the basis of the amount of sudanophil lipid observed, a clear cut correlation was found. It is not intended that this evidence should serve as a basis for the generalization that in the liver a high sudanophil content necessarily implies a high fat content. It is known that in the fatty liver of chloroform and arsenical poisoning there is no comparable increase in total lipid (22).

The liver phospholipid percentages classified on a similar basis gave no such regularity; the average percentages were for "abundant" 3.4, for "moderate" 3.6, and for "scanty" 3.1.

On the basis of the histological classification, the division of the neutral fat averages is striking (Table VIII) and is evidence that the Sudan IV stain is specific for neutral fat. The average neutral fat content of those classed as "moderate" is 6 times the average of those called "scanty;" the "abundant" group has 33 times as much neutral fat as the "scanty" group.

#### DISCUSSION

Fatty livers have been produced by a variety of agents such as drugs, diseases, fat or cholesterol feeding (in rabbits), and toxins. This leads to the concept of the fatty liver as a pathological condition arising from pathological processes. In contrast, the production of fatty liver by fasting would seem to be a physiological process; certainly every wild animal has involuntary fasting periods. Mechanisms to meet acute nutritional deprivations, among which the mobilization of fats is of great importance, should be normal mechanisms. The various fat changes described above are given additional interest and importance because they arise from "physiological" stimuli. The mouse carcass, after 4 days of fasting, has only 20 per cent of its original lipid, but the remaining lipid is to be viewed as essential and in no way as pathological. The mouse liver, after 4 days of fasting, has lost one-half its original substance; yet the concentrations of the various lipids are essentially as they were before fasting. Massive, almost dramatic fatty changes have occurred in the fasting period but the stimuli which initiated them are normal ones and the inference is that the mechanisms, modes of action, and changes are exaggerated but essentially normal processes.

The 3 month-old mouse is able to burn all its available body fat in 48 hours. The rapidity of removal of depot fat is to be ascribed first, to the fact that the 3 month-old mouse is still growing and presumably has the higher metabolic rate of young animals, and, second, to the known high activity and high metabolic rate (per kilo of body weight) of mice. Still, the burning of 0.8 gm. of fat per day seems a remarkable performance; on a weight for weight basis a 70 kilo man would have to burn 2.8 kilos of fat,

equivalent to approximately 25,000 calories, per day. This excessive fat catabolism must be regarded as largely wasted from the standpoint of energy metabolism, since the burning of 0.8 gm. of fat would produce 7 calories, whereas the basal requirement of the mouse is probably of the order of 3 calories per day (23).

*Mobilization*—Under the physiological stimulus of fasting, the young adult white mouse is able to mobilize its fat fuel reserves with unexpected rapidity and to a surprising extent. Of its 2 gm. of body fat, 80 per cent disappears, the major part certainly burned, in 2 days, one-half each day. The fat is taken from the depots without selection as to degree of saturation of the fatty acids. The fat burned probably has an average iodine number of about 80, which is a little higher than that of the depot fat in most mammals (about 60). Along with the fat, about 8 mg. of cholesterol are mobilized; this amounts to one-sixth of the total in the body. Left in the mouse's body (exclusive of the liver) at the end of 2 days fasting is a minimum (0.4 gm.) amount of fatty material which is apparently essential (structural or functional?), because 2 days additional fasting reduces it only slightly.

*Transport*—There is no evidence in these data as to modes or rates of transport. However, the ability of the transport mechanism to carry such relatively large loads of lipids is noteworthy. It is improbable that the mouse usually ingests amounts of fat large enough to incur a comparable transport problem.

Barrett *et al.* (9), using deuterium-labeled fats, have observed that the deuterium content of the fat found in the liver during fasting in certain cases approaches that of the depot fat. The hypothesis that the liver is the destination of the mobilized depot fat is supported by two facts from the present data: The depot fat is transported (one-half each day) during a 2 day period in which the cholesterol content of the liver increases by approximately equal daily increments. The increase in liver cholesterol leads to the speculation that the mobilized fat on each day passed through the liver. The second fact which may be interpreted as indicating that all the metabolized fat goes through the liver is the nearly linear relation between the amount of carcass fat and the amount of total liver fat.

*Liver Fat Metabolism*—The liver neutral fat increases strikingly

(average, 300 per cent) after 1 day's fasting and, histologically, the liver shows massive fatty infiltration. However, this does not indicate at all that the liver is functionally seriously damaged. In fact, 92 per cent of the depot fat taken to the liver on the 1st day is metabolized; only 8 per cent remains as excess neutral fat in the liver. As further evidence of the unimpaired condition of the liver, on the 2nd day's fasting, the liver not only metabolizes all the depot fat mobilized that day (as much as on the 1st day) but also about four-fifths of the excess neutral fat from the 1st day. The liver also shows an almost normal content of sudanophil substances. Whether this can be ascribed to the adjustment of the organism to the economy of fasting and whether specific lipotropic substances are brought to the liver as the result of body protein utilization are interesting speculations.

An important point is the "factor of safety" of the liver. The appearance of neutral fat after 1 day's fasting shows that the safety factor can be exceeded. Apparently the mobilized depot fat temporarily overloads the various mechanisms of the liver for handling fat, *e.g.* ester-splitting, phosphorylating, desaturating, and fragmenting, so that neutral fat piles up. However, by the end of the 2nd day, 99.5 per cent of the total depot fat taken to the liver has disappeared; only 0.5 per cent remains as excess neutral fat. This constitutes a notable adaptability to the necessity of almost exclusive fat combustion.

If all the depot fat is brought to the liver and there converted to phospholipid in preparation for burning, the liver would be required to produce 30 times its normal content of phospholipid on the 1st and also on the 2nd day of fasting. The ability of enzymatic processes to accomplish such increases is not at all doubted. However, it seems plausible that if the liver produced so much phospholipid on each of the first 2 days and practically none at all on the 3rd and 4th days, there might well be some variation in the amount of phospholipid in the liver from day to day. The demonstrated constancy of phospholipid concentration gives rise to the inference that a major part of the depot fat may be metabolized without phosphorylation; the metabolic route preferred may well be through the formation of ketone bodies. In any event, the phospholipid (if formed) must be burned as rapidly as it is formed, since, on the 3rd day, there is no increase of liver

phospholipid over normal and there is a minimal amount of lipid in the carcass. There is no evidence here for an "interchange of fat" between the liver and the depots (9).

The liver of the fasted mouse (after 4 days) is about one-half the normal size but, except for neutral fat, it has a nearly normal lipid distribution. This constancy of phospholipid and cholesterol percentages raises the hypothesis that these liver lipids should be considered essential in the amounts in which they normally occur. The neutral fat is present in uniform amounts in the normal liver; it may be mainly a true "metabolic" lipid, since most of it disappears in a number of cases. The remaining lipids all seem to be included in the *élément constant* of Mayer and Schaeffer, whether this is to be interpreted as evidence of a structural rôle or not. One-half of the original phospholipid and cholesterol has disappeared along with one-half the liver, but the liver of the fasted mouse has nearly normal distributions of these substances.

#### SUMMARY

1. Under the conditions of the experiment, the 3 month-old male albino mouse exhibited a high fasting mortality rate; *viz.*, 70 per cent in 4 days. The body weight loss (30 per cent) for such a high mortality is low in comparison to that of many other mammals. The utilizable carcass lipids are mobilized and disappear in 2 days; roughly one-half of the total originally present is lost each day. There is a linear relation between the logarithm of the amount of total carcass lipids and their iodine number. On the assumption that the iodine number of the utilizable carcass lipid is about 80 and of the essential lipid about 100 to 110, it can be shown that there is no evidence of a selective utilization of the more unsaturated fatty acids. The fasting mouse loses about 1 gm. per day of non-lipid constituents; in addition, on each of the first 2 days, 1 gm. of fat is lost.

2. The liver loses about one-half its weight in 2 days, after which there is no further loss. In contrast, the body loses only 20 per cent of its weight in 2 days and thereafter loses an additional 10 per cent. The liver *total lipid* increases 2- or 3-fold in the 1st day of fasting, and decreases to normal on the 2nd day and to about one-half its original content on the 3rd and 4th days. The



total lipid increase is marked; twenty-one mice had 10 per cent or more and one mouse had 26 per cent lipid (moist weight basis). The liver *phospholipid* decreases to one-half of its original absolute amount but undergoes no change in concentration. The liver *neutral fat* increases by 300 per cent in 1 day and thereafter decreases to about one-half its original amount on the 3rd and 4th days. The amount of neutral fat in the liver in fasting is closely dependent on the amount of body fat remaining in the carcass. About 8 mg. of total cholesterol are lost from the carcass on fasting. Of this about 1 mg. appears in the liver as *ester cholesterol*; the remainder is probably burned or excreted. On the 3rd and 4th fasting days, 2 mg. of cholesterol are lost from the liver.

3. The liver metabolizes 92 per cent of 800 mg. of depot fat on the 1st day of fasting; the balance (8 per cent) remains as sudanophil neutral fat. On the 2nd day, the liver metabolizes 100 per cent of an additional 800 mg. of depot fat without trace (histologically) and also removes nearly all the residual neutral fat remaining on the 1st day. In its lipid distribution the liver of the fasted mouse is like the normal liver.

4. Sudanophil lipid was seen in all normal livers and, on the average, was abnormally abundant after 1 day of fasting and abnormally scanty after 3 or 4 days of fasting. The sudanophil content of liver is closely related to the neutral fat content; no such relation exists for phospholipid or cholesterol. The result of the Schultz cholesterol test was negative for all the normal livers and almost invariably negative for the livers from the fasted mice.

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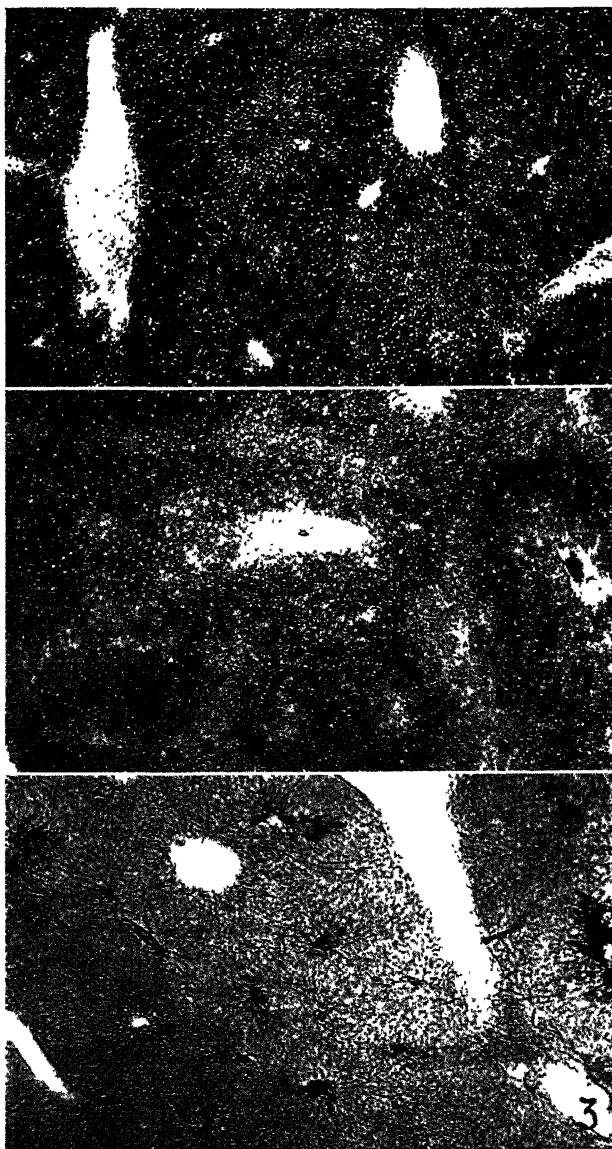
## EXPLANATION OF PLATE 6

Photomicrographs of frozen sections of mouse livers stained with Sudan IV only. The lipid appears black or dark gray, the liver tissue light gray.  $\times 50$ . (Photographs by M. C. Orser.)

FIG. 1. The lipid is abundant, occupying all the liver tissue. From a mouse fasted for 1 day.

FIG. 2. The lipid is moderately abundant, giving the liver a mottled appearance. From a normal mouse.

FIG. 3. The lipid is scanty, the cells containing it occurring in groups. From a mouse fasted 4 days.



(Hodge, MacLachlan, Bloor, Stoneburg, Oleson, and Whitehead: Lipids of fasting mouse)



## THE INABILITY OF CREATINE AND CREATININE TO ENTER INTO TRANSMETHYLATION IN VIVO

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By the labeling of the S-methyl group of methionine with deuterium it was demonstrated that methionine can furnish the methyl groups of creatine in muscle (1) and of the creatinine excreted in the urine.<sup>1</sup> A thorough investigation of the possible reversibility of this process seemed warranted.

One experimental approach to this question would be to reverse the procedure of the above investigation; that is, to feed creatine or creatinine containing a deuteromethyl group and then ascertain whether this labeled methyl group could be transferred to the methionine of the tissues. This attack is now being undertaken in this laboratory. However, another method of attack has also been employed, the results of which we wish to report in the present communication. In this study we have tested the ability of creatine and creatinine to act as methyl donators when fed to animals kept on a diet containing homocystine but free of methionine and such methyl donators as choline and betaine. With such a "biologically labile" methyl-free diet it was shown that the administration of choline allowed homocystine to replace methionine (2). This evidence led to the conclusion that homocystine was converted to methionine by methyl transfer<sup>2</sup> and demonstrated the inability of the body to generate methyl groups for this methylation. A preliminary experiment was also reported in which creatinine, unlike choline, could not function as a methyl

<sup>1</sup> du Vigneaud, V., Schenck, J. R., and Simmonds, S., unpublished data.

<sup>2</sup> Direct evidence has recently been obtained in this laboratory for this methyl transfer by the labeling of the methyl group with deuterium.

donator when fed at a level containing an amount of methyl groups equivalent to that of an effective dose of choline. In the present investigation not only creatinine but also creatine has been tested, at levels considerably higher than those used in the preliminary test of creatinine just referred to. Since creatinine does not appear to be convertible to creatine in the rat (3), the testing of creatine itself was necessary. Such a study seemed particularly desirable in view of Griffith and Mulford's report (4) that creatine exerted a protective action against hemorrhagic kidneys in rats fed a diet low in choline.

Sarcosine has also been included in this study. Since evidence has been presented by Abbott and Lewis (5) and by Bloch and Schoenheimer (6) that sarcosine may be demethylated to glycine, it was of particular interest to see whether in this demethylation the methyl group is removed in such a manner that it might enter into transmethylation.

#### EXPERIMENTAL

Two litters of albino rats 27 and 30 days of age, respectively, were placed on a basal diet which contained homocystine but was free of methionine. The diet and vitamin supplements had the same composition as those described in a previous communication (7), except that both pyridoxine (10  $\gamma$  per day) and choline-free ryzamin-B were included in the vitamin pills and that 2-methylnaphthoquinone (5  $\gamma$  per day) was included in the diet. The creatine, creatinine, or sarcosine was mixed in the diet and an equal weight of dextrin removed therefrom. When choline chloride was fed, it was incorporated in the vitamin pills. This methionine-deficient diet has been shown to be adequate for the support of growth only with the addition of methionine or of a compound such as choline which will furnish methyl groups to convert homocystine into methionine (2).

Of the eight rats of Litter I, four were fed the basal diet described above and the other four were given the diet containing 4 per cent creatine. All of the rats immediately lost weight, and one of those receiving creatine died on the 9th day. Upon autopsy the latter was found to have greatly enlarged and hemorrhagic kidneys. On the 11th day 5 mg. of choline chloride were added to the daily vitamin supplement of two rats, Nos. 485 and 486, receiving

is given in Table I. Creatine has no apparent effect on the growth rate of the animals. It will be noted also that there was no growth in the absence of choline but that a moderate growth rate was maintained in all cases in which the 5 mg. of choline chloride were added to the supplement.



Litter II consisted of nine rats, four of which received the basal diet. Three rats were fed the basal diet to which had been added, respectively, 4, 2, and 1 per cent creatinine. The two remaining rats were fed the diet containing 4 per cent sarcosine. Two of the

TABLE I  
*Data on Food Consumption*

Litter No.	Rat No. and sex	Days	Daily supplement	Average daily food consumption
				<i>gm.</i>
I	485 ♂	1-11	108 mg. creatine	2.7
		11-35	42 " " + 5 mg. choline chloride	4.2
		35-44	5 mg. choline chloride	4.6
	486 ♀	1-11	104 " creatine	2.6
		11-43	40 " " + 5 mg. choline chloride	4.0
	487 ♂	1-11	116 mg. creatine	2.9
		11-44	42 " "	4.2
	488 ♂	1- 9*	64 " "	1.6
	489 ♂	1-11	No supplement	3.2
		11-44	5 mg. choline chloride	4.2
	490 ♀	1-11	No supplement	3.3
		11-46	5 mg. choline chloride	4.5
	491 ♂	1-25	No supplement	4.0
	492 ♀	1-44	" "	4.0
II	500 ♂	1-23	50 mg. choline chloride	4.4
	501 ♂	1- 7*	No supplement	3.3
	502 ♂	1-34	" "	4.7
	503 ♂	1-35	116 mg. creatinine	2.9
	504 ♀	1-35	82 " "	4.1
	505 ♀	1-12	50 " choline chloride	3.9
		12-35	25 " " "	5.8
	506 ♀	1-35	39 " creatinine	3.9
	507 ♂	1-34	152 " sarcosine	3.8
	508 ♀	1-34	168 " "	4.2

\* Died.

rats on the basal diet received choline chloride in the vitamin supplements in the amounts shown in Table I. One of the two on the basal diet without choline died on the 7th day, and upon autopsy was found to have greatly enlarged and hemorrhagic

kidneys. The growth curves of Litter II are shown in Fig. 2 and the food consumption is given in Table I. Growth followed the administration of choline but neither creatinine nor sarcosine was effective in this respect.

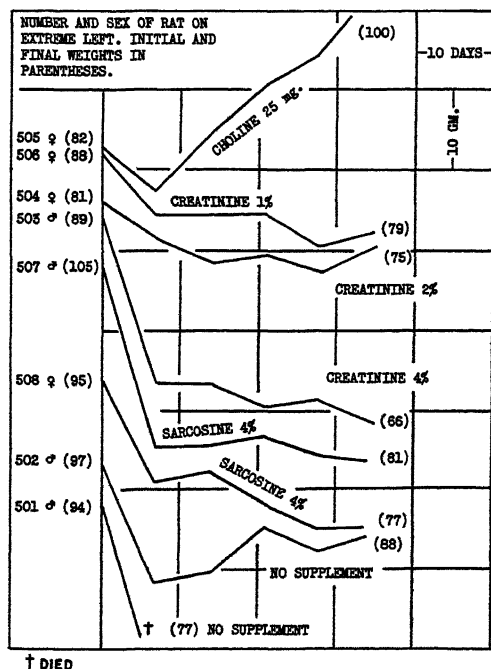


FIG. 2. Growth curves of Litter II showing comparative effects of feeding choline, creatinine, and sarcosine. The levels of creatinine and sarcosine are indicated as per cent composition of the diet; the choline level is indicated as mg. per day. Rat 505 received 50 mg. of choline chloride during the first 12 days of the experiment. To conserve space the growth curve of Rat 500, similar to that of Rat 505, has been omitted.

Results agreeing with those described above were obtained in some preliminary experiments with rats on a similar diet. In two instances, creatinine at levels of 50 and 100 mg. daily, respectively, did not produce growth. 50 mg. of sarcosine in two experiments and 50 mg. of creatine in one experiment were without effect.

Determinations of liver fat, according to the method of Best,

Channon, and Ridout (8) were made after sacrifice of the animals. In all cases the fat content of the livers was high (25 to 40 per cent of the wet weight) and no lipotropic effect due to the feeding of creatine, creatinine, or sarcosine was observed.

#### DISCUSSION

It is apparent from the results described above that creatine and creatinine are not sources of available methyl groups for the methylation of homocystine in the rat, even though fed in quantities containing methyl groups several fold the amount necessary when given in the form of choline. Since methionine has previously been shown to furnish methyl groups for the synthesis of creatine (1) and creatinine,<sup>1</sup> the shift of the methyl group in this instance is irreversible. If methyl groups of creatine or of creatinine were available, as those of choline are available for the methylation of other compounds, then the methionine should have been formed from homocystine and growth would have resulted.

It also follows from this evidence that administration of creatinine or of creatine did not lead to choline synthesis. If this had occurred, the resulting choline would have made growth possible by furnishing methyl groups for the synthesis of methionine, since choline itself has been shown to behave in this fashion when administered under these dietary conditions.

In the recent report of Griffith and Mulford (4) that creatine is only partially effective in the prevention of hemorrhagic kidneys in rats on a choline-deficient diet, the limited action of creatine is ascribed, not to the limited availability of the methyl group of creatine for choline synthesis, but to a sparing action. Our investigations discussed above provide additional experimental proof in support of this interpretation. As already pointed out, the feeding of deuteromethylmethionine (1) and deuteromethylcholine<sup>1</sup> has shown that the methyl group can be transferred from methionine or choline to creatine. It is possible that the feeding of creatine might, under circumstances in which the available supply of "biologically labile" methyl groups is limited, spare the body from expending as much of this "labile" methyl supply as would ordinarily be used in the synthesis of creatine. In this manner the body might conserve more efficiently the methyl groups

needed to provide sufficient choline to protect against hemorrhagic kidney. It should be borne in mind that, in the experiment of Griffith and Mulford, methionine and undoubtedly some choline were present in the diet, so that a sparing action might well become evident, whereas in our diet, free of such methyl groups, no effect of creatine would likely be manifested unless the methyl groups were labile and available for transmethylation. By the same token we would not expect as much sparing action to be shown under our dietary régime even on kidney damage.

It will be noted that creatine and creatinine exerted no protection against the fatty infiltration of the liver, which confirms a similar finding on creatine reported by Platt (9). Griffith and Mulford reported no significant lipotropic activity on the part of creatine unless both creatine and choline were fed together.

The methyl group of sarcosine, although removable by the body (5, 6), is apparently not available for supplying the methyl groups for the synthesis of choline or methionine.

#### SUMMARY

Creatine, creatinine, or sarcosine does not enable homocystine to be used in the diet in lieu of methionine for the growth of rats. It is concluded that these compounds do not give up methyl groups for the methylation of homocystine to make methionine.

These findings are discussed in relation to the participation of labile methyl groups in general metabolic processes.

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## ISOLATION AND PROPERTIES OF PIGMENTED HEAVY PARTICLES FROM STREPTOCOCCUS PYOGENES\*

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During the past few years, a number of macromolecular materials have been isolated, with the aid of high speed centrifuges, from healthy and diseased plant and animal tissues (Wyckoff (1), Claude (2), Henle and Chambers (3), Smadel *et al.* (4), and Rivers (5)). While nucleoproteins invariably form the "base" of these complex macromolecules, other constituents such as lipids, carbohydrates, pigments, and enzymes have also been demonstrated in some of them. Some of these materials have been found to possess characteristic biological properties and have been identified with certain animal and plant viruses (Stanley (6)). No comprehensive studies have as yet been made on the presence of similar macromolecules in unicellular organisms.

French (7) has been able to isolate chlorophyll-protein complexes from certain photosynthetic purple bacteria (*e.g.* *Rhodospseudomonas* or *Phaeomonas varians*, *Rhodospirillum rubrum*, and *Rhodovibrio* sp.). However, their molecular weight is unknown and might well fall within the range of the ordinary, relatively low molecular weight proteins since they yield clear solutions.

The present report deals with the isolation and various properties of heavy particles prepared from two strains of *Streptococcus pyogenes*. Apart from its large particle size, this material invites more detailed studies in view of its highly complex chemical composition, which includes lipid, nucleic acid, protein, carbohydrate,

\* This work was conducted at the University of Pennsylvania under a grant from the Commonwealth Fund and at Yale University with the aid of a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

and a green pigment. The evidence presented in this communication appears to justify consideration of these heavy particles as being macromolecular<sup>1</sup> in nature.

## EXPERIMENTAL

### *Material*

*Streptococcus pyogenes* cultures were grown for 16 to 18 hours in 50 liter batches in meat extract broth containing 1 per cent peptone. Strains 1048 mucoid (Type 6) and 1685 mucoid (Type 1) were employed. The former strain served for most of the experiments.

### *Methods for Isolation of Macromolecular Material*

The first step in the isolation of the macromolecular material consisted in the disintegration of the bacterial cell. This was accomplished effectively by subjecting the organisms to intense sonic vibration, or crushing in a suitable mill. In the majority of the present experiments the bacteria were collected from the culture medium by centrifugation. Concentrated suspensions in saline or in phosphate buffer were then subjected to the action of waves of sonic frequency in a magnetostriction oscillator (8, 9), which is patterned rather closely after the original design of Gaines (9). For these sonic treatments the authors are greatly indebted to Dr. Leslie A. Chambers and Dr. H. E. Calkins of the Johnson Foundation for Medical Physics. During the treatment, the suspension was in contact with the water-cooled vibrating nickel tube for a period of 60 minutes. In a few experiments, the low temperature ball mill (10) was employed for the same purpose with

<sup>1</sup> By "macromolecular material" we mean particles of large size whose constituents are not separated by the several physical means described in detail in this paper. The particles are of complex composition. They are not homogeneous in size (see Table II), and we have as yet no final evidence as to whether the larger ones are polymers of the smaller or even whether or not each macromolecule is identical with each other macromolecule in chemical composition. If continued investigation reveals that no other physical means of separation can resolve the heavy particles into their components, it will finally have been proved that use of the term "molecule" is appropriate. We believe present evidence to justify suggestion of the term at the present time.

similar results. Part of the cellular contents was thereby released into the suspension fluid, while a large portion of the bacterial mass was left in insoluble form. The débris, together with those cells which escaped the disintegration, was separated from the "sonic" or ball mill extract in the centrifuge and by filtration through an E. K. Seitz filter. The solid residue could be further extracted with saline or phosphate buffers. For purposes of preservation and concentration of bacterial suspensions, as well as sonic extracts, the cryochem and desivac drying apparatus (11) were employed extensively in this work.

The extracts on concentration, as well as the solid residues even after repeated extraction with aqueous solvents, invariably were green in color. An association of the pigment with a protein was indicated by the fact that when 2 volumes of saturated ammonium sulfate solution were added to the bacterial extracts at pH 7.1 both pigment and protein were precipitated. The precipitate could be resuspended in distilled water and dialyzed against running water for 48 hours without loss in coloring matter. The dialyzed solutions were then dried by the cryochem process. In two experiments the solutions were concentrated by being placed in cellophane bags and exposed to a current of air from an electric fan (12).

Crude sonic or ball mill extracts of *Streptococcus pyogenes* as well as preparations partially purified by ammonium sulfate precipitation, when subjected to gravitational fields of 60,000 to 90,000 *g* for 1 hour in the chilled concentration rotor of a Beams air-driven ultracentrifuge (13), yield dark green translucent pellets. If sufficient time has been allowed for sedimentation, the supernatant solution is practically devoid of green pigment. The pellets are readily resuspended in dilute phosphate buffer (0.017 to 0.1 M) at pH 7.3. Some insoluble colorless material may be removed by centrifugation in the angle head of an International centrifuge (20 minutes at 3500 R.P.M.). The supernatant solution is now practically transparent to transmitted light but it shows an appreciable opalescence when viewed in reflected light. The green, macromolecular material may be further purified by repeating the cycle consisting of high speed and subsequent low speed centrifugation. It has been found, however, that this process cannot be repeated more than two to three times without a breakdown of the



complex. Such apparently irreversible damage manifests itself in a marked loss in solubility of the pellets sedimented in the ultracentrifuge and the separation of colorless protein with diminished solubility. Consequently, the number of high speed centrifugations was later limited to two. Before and after each ultracentrifugation, aggregated matter was removed by low speed centrifuging.

In order to illustrate the technique finally adopted, one preparation (Tables I and II, Pellet 18) will be described in detail. The bacteria harvested from 450 liters of culture medium were suspended in phosphate buffer at pH 7.2 and were disintegrated by sonic vibration. The "sonic" extract thus obtained was filtered through a Seitz filter, dialyzed, and then dried from the frozen state by the cryochem process. The yield of dry material was 12.64 gm. This material was dissolved in 360 cc. of distilled water and treated with 2 volumes of saturated ammonium sulfate solution at pH 7. After the mixture had stood for 20 hours in the refrigerator, the dark green precipitate was collected in the angle centrifuge. It was redissolved in distilled water and dialyzed against running distilled water to remove residual ammonium sulfate. In this manner 230 cc. of an olive-brown solution, containing 2.576 gm. of solids, were obtained. The solution was spun in lusteroid tubes in the chilled quantity rotor of the air-driven ultracentrifuge for 75 minutes at 36,000 R.P.M. (maximum field, 90,000 *g*) and green, translucent pellets were obtained at the bottom of the tubes. The supernatant fluid was carefully decanted and the pellets first rapidly rinsed with and then pooled and dissolved in 60 cc. of 0.017 M phosphate buffer of pH 7.3. The resultant opalescent, olive-green solution was stored overnight in the refrigerator. It was clarified by spinning in the angle head of the International centrifuge for 20 minutes at 3500 R.P.M. A trace of brownish sediment was discarded. The supernatant solution was diluted to 100 cc. with phosphate buffer and once more ultracentrifuged for 75 minutes at 36,000 R.P.M. The green pellets thus obtained were again rinsed with and then dissolved in 0.017 M phosphate buffer at pH 7.3. Subsequent low speed centrifugation did not remove any visible sediment. The supernatant green solution had a volume of 40 cc. and contained 510 mg. of macromolecular material. Since the mass of bacteria con-

tained in 450 liters of culture is approximately 120 gm., the yield obtained here amounted to 0.43 per cent.

It should be mentioned that in control experiments, in which uninoculated peptone-extract broth and culture supernatant filtered through a Seitz filter were ultracentrifuged under the same conditions, neither green pellets nor any other heavy material was obtained. On the other hand, dialyzed and non-dialyzed sonic and ball mill extracts of Strains 1048M and 1685M, as well as the solutions of the precipitates obtained at 66 per cent saturation with ammonium sulfate, invariably produced the green macromolecular material upon high speed centrifugation. These observations indicate that we are not dealing with an artifact arising during the manipulation of the preparations, but, on the contrary, with an intrinsic constituent of these bacteria.

### *Chemical Studies*

Chemical and serological evidence has indicated the presence and relative amounts of lipid, protein, nucleic acid, and carbohydrate in the purified macromolecular material isolated from *Streptococcus pyogenes*, as well as the green pigment which is responsible for the color. As an example, the examination of the material designated as Pellet 18 (Table I) yielded the following results.

The purified solution was dialyzed against running, distilled water for 48 hours and then dried from the frozen state. The dry product was found to contain 9.9 per cent total nitrogen, 0.73 per cent purine nitrogen (14), and 1.01 per cent phosphorus. For the determination of total lipid present, 97.5 mg. of the dried product were first extracted with 50 cc. of absolute ethyl ether and then with 20 cc. of a mixture of equal parts of ether and ethyl alcohol. Upon evaporation of the solvents of the combined extracts and subsequent drying at 110°, a residue weighing 17.4 mg. was obtained. When this was corrected for the blank value (0.5 mg.), a lipid content of 17.2 per cent was calculated. In order to characterize the nucleic acid present in the material the following qualitative tests were conducted, with suitable positive and negative control solutions. The orcinol test for pentoses was positive; the diphenylamine and the Feulgen tests for desoxyribose were negative. These results, when taken together with the purine

nitrogen and phosphorus content of the material, indicate that there is present a nucleic acid of the *d*-ribose type. Analytical data on this and similar preparations will be found in Table I.

TABLE I  
Data on Preparations Isolated from *Streptococcus pyogenes*,  
Strain 1048M (Type 6)

Culture	Dry weight of streptococci (approximate)	Weight of extracted material (non-dialyzable)		Pellet No.	Yield per 100 gm. dry streptococci (approximate)	Total N	P	Purine N	Lipid
		Ball mill	Sonic						
liters	gm.	gm.	gm.		mg.	per cent	per cent	per cent	per cent
43	8.6	0.68	0.74	1					
78	15.6		0.55	8	150				
42	8.5			9	200				
67	13.4		1.30	11	600	9.0	0.82	0.84	10*
			1.12	12-14	420	9.0	0.82		
31	9.0		0.83	16	730	10.1	0.96		10*
34	9.2		1.13	17	380				
450	120.0		12.64	18	450	9.9	1.01	0.73	17.2†
200	40.0		10.68	19	880				
250	50.0		17.20	20‡	1400	10.0	1.4	0.4	23.0†

The source of the Pellets 1, 12-14, 19, and 20 was sonic extract; the source of Pellets 8, 9, 11, 16, 17, and 18 was 0.66 saturated  $(\text{NH}_4)_2\text{SO}_4$  fraction of sonic extracts.

\* Lipids extracted with absolute ethyl ether.

† Lipids first extracted with absolute ethyl ether followed by a second extraction with a mixture of equal parts of ethyl ether and absolute ethyl alcohol.

‡ Pellet 20 was purified by being twice ultracentrifuged from 0.017 M phosphate buffer of pH 7.3. It was then precipitated twice from the buffer solution of pH 7.3 with 66 per cent saturated neutral  $(\text{NH}_4)_2\text{SO}_4$  solution. The analysis before and after  $(\text{NH}_4)_2\text{SO}_4$  precipitation remained unchanged. High percentage of phosphorus in certain preparations in comparison to purine nitrogen indicates that they contain, besides the nucleic acid, another phosphorus-containing and purine nitrogen-free component. We cannot at this time account for this difference.

*Heavy Metals*—The demonstration of the presence or absence of certain heavy metals in such pigmented macromolecular materials would be of interest in a possible correlation of the green pigment with the known biological pigments. By determination

of the absorption spectra of the solution of macromolecular material nothing characteristic of heme or chlorophyll type of pigments has been found. This would appear to indicate that the color cannot be accounted for by Fe or Mg in such combination. Another consideration is the presence of nickel as an impurity. Inasmuch as the sonic treatment of the bacteria brings the cell suspension into contact with the nickel tube vibrator, our original extracts might be contaminated with metallic nickel. However, various steps used in the purification of the macromolecular material should eliminate this impurity. Nevertheless, tests were carried out for the presence of this and other cations as follows:

4 cc. of solution of 0.017 M phosphate buffer of pH 7.3 containing 52 mg. of purified macromolecular material (Pellet 18) were dried and ashed in a quartz crucible. The ash was dissolved in 2 cc. of 0.1 N HCl. Of this solution 0.8 cc. was tested for Cu with rhodanine, and 0.3 cc. aliquots of solution in volumes of 3 cc. were tested for Fe with potassium ferrocyanide, and also with ammonium thiocyanate; 0.3 cc. in 3 cc. for Mg with sodium phosphate in weak ammonium hydroxide solution; 0.3 cc. in 3 cc. for Ni with 1 per cent alcoholic solution of dimethylglyoxime. The tests were negative for the metals considered above.

Assuming the minimum molecular weight of  $10^6$  and the sensitivities of each of the tests for the cations per cc. to be for copper 1  $\gamma$ , iron 0.07  $\gamma$ , magnesium 0.05  $\gamma$ , and nickel 0.2  $\gamma$ , then the negative results would permit the following conclusions. If any of these metals is present at all, there cannot be more than approximately 25, 4, and 1 atom, respectively, each of copper, iron, and magnesium per single macromolecule. Furthermore, as impurity a maximum of 0.001 per cent of nickel could be present. Accordingly, it seems doubtful whether any chromophore grouping containing the above metals could be responsible for the pigmentation of such intensity, and most certainly the color cannot be due to contamination by nickel ions.

*Serological Data on Presence of Carbohydrate in Macromolecular Material*—The sera of rabbits immunized against macromolecular material (Pellets 18 and 19) gave precipitates with the macromolecular substance in dilutions of 1:50,000. These sera reacted with two different preparations of the group carbohydrate from *Streptococcus pyogenes* in dilutions up to 1:100,000. Further

serological results including those from absorption experiments will be reported later.

*Pigment*—It has as yet not been possible to identify the green pigment associated with the macromolecular material. Neither direct observation of the purified preparations with the pocket spectroscope nor analysis of such solutions, in the visible region of

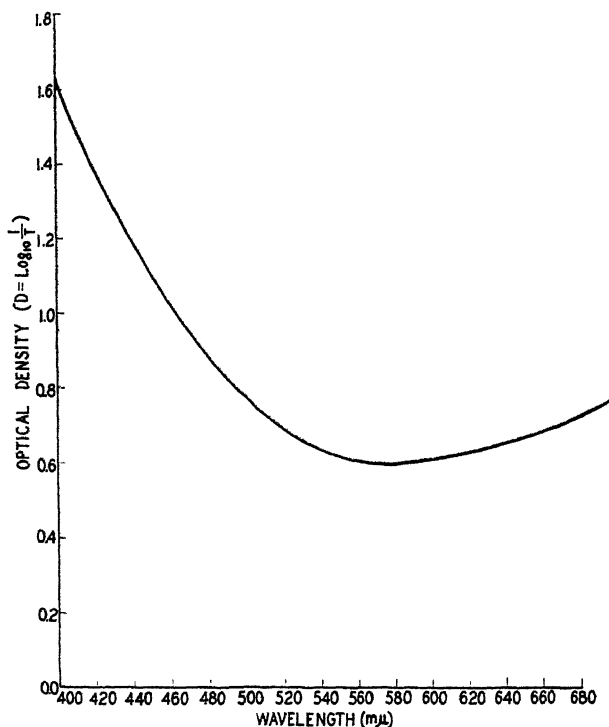


FIG. 1. The absorption curve in the visible region of the pigmented heavy particles as recorded with Hardy's spectrophotometer.

the spectrum, with Hardy's recording spectrophotometer has revealed the presence of absorption maxima between 400 and 700 mμ. The absorption curve has a minimum near 580 mμ<sup>2</sup> (see Fig. 1).

<sup>2</sup> Recorded by Dr. S. Q. Duntley of the Massachusetts Institute of Technology, with Hardy's recording spectrophotometer.

The nature of the absorption in the visible region does not permit a direct comparison with known biological pigments. The green color is explained by the presence of maximum transmission in the region of  $\lambda$  580  $m\mu$ . Tests performed suggest that French's bacterial chlorophyll is not responsible for the absorption curve obtained, although the material seems to absorb in the near infrared (800 to 900  $m\mu$ ). Another pigment considered was Lemberg's (15) verdohemochromogen, but we have no reason to believe that such a pigment might be responsible. The nature of the pigment, therefore, is unknown.

Spectroscopic and chromatographic tests on a pigmented material, extracted from the green, insoluble residues of sonic bacterial extracts with alcohol and ether, were equally inconclusive.

### *Physicochemical Studies*

*Examination in Analytical Ultracentrifuge*—A number of preparations of the macromolecular material, which had been purified to varying degrees, were examined in a Beams air-driven ultracentrifuge, arranged for optical studies by Svedberg's light absorption method (Wyckoff (1)). It was found that the contrast in the photographic records could be considerably improved by omitting the chlorine filter and employing the bromine filter alone which transmits ultraviolet radiation up to about 380  $m\mu$ . The opacity of the sedimenting material, under these conditions, is due to light scattering as well as to light absorption. In all instances, only one boundary was recorded on the diagrams, the definition of which decreased with the distance sedimented in the cell. In the early stages of sedimentation the boundary was usually so well defined that its displacement with time could be measured directly to 0.1 mm. with a Bausch and Lomb lens magnifier with a built-in scale. As a rule, the films were examined with the recording photoelectric microphotometer of Koch-Goos. The shift in the position of the mid-points of the photometer tracings of the individual boundaries was considered to represent the rate at which the particles of average size in a population of particles of somewhat differing, but continuously overlapping, dimensions were settling in the gravitational field employed. From the character of the diagrams obtained (see Figs. 2 and 3) it is obvious that the material is polydisperse. Moreover the sedimentation constants derived from

these records showed an appreciable variation from experiment to experiment. The data obtained in these analytical runs are compiled in Table II.

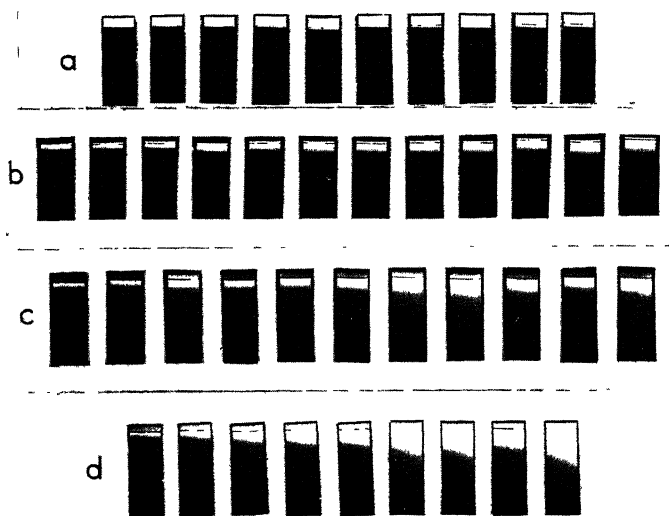


FIG. 2. Sedimentation diagrams of macromolecular preparations from *Streptococcus* obtained in the ultracentrifuge by the light absorption method. Mercury high pressure arc, bromine filter, Eastman positive film, 3 mm. thick fluid cell, speed 120 revolutions per second =  $3470 \times g$ . The fluctuation in density in series *c* is due to variations in light intensity during the run due to stroboscopic phenomena. The different series of diagrams were obtained with different batches of purified pellet material. These diagrams were selected because they appeared representative of the diagrams obtained in this work. They were particularly meant to bring out the point mentioned in the text that the boundaries were usually well defined during the early stages of sedimentation but that later considerable blurring due to the size and inhomogeneity of the material took place, leading eventually to a "disintegration" of the boundary.

The density of the macromolecular material was found to be 1.22 with the aid of the falling drop method of Barbour and Hamilton.<sup>3</sup> Since the material did not display flow birefringence when agitated between crossed polaroid disks, the particles are probably not

<sup>3</sup> For this determination, the authors are indebted to Mr. Paul Barbour, Jr.

very asymmetric. Assuming, then, the validity of Stokes' law for the material under study, the diameters listed in Table II were calculated. It must be borne in mind, however, that these values represent at best the mean diameter of the majority of the particles present in the individual preparations. The mean value for all preparations is 42  $m\mu$ . A spherical particle of this diameter

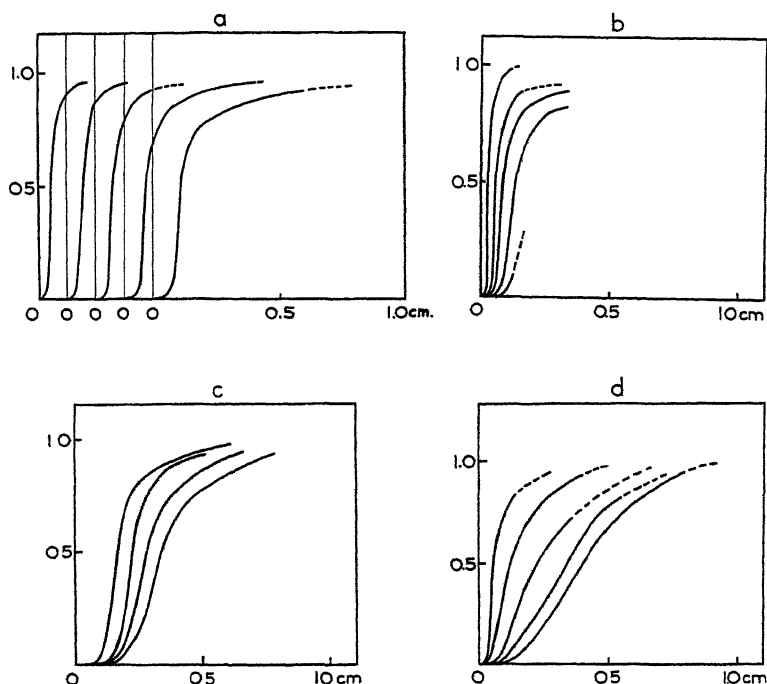


Fig. 3. Microphotometer tracings of sedimenting boundaries of macromolecular material isolated from *Streptococcus pyogenes*. Ordinates, approximate relative concentration; abscissae, distance from the center of rotation.

and density<sup>4</sup> of 1.25 would have a weight of  $4.8 \times 10^{-17}$  gm. From this there follows a "molecular" weight of 29 million.

<sup>4</sup> The density, as actually determined, was 1.22. To calculate the weight of the individual particles and also the "molecular" weight, graphs based on density values of 1.25, were employed. Inasmuch as our values can at best be very approximate, considering the heterodispersity of the material, refinement in the calculations was considered superfluous.



TABLE II

*Sedimentation Data on Macromolecular Chromoprotein from Streptococcus pyogenes (Strain 1048M)*

Preparation No.	Pellet No.*	Preparation for sedimentation	$S_{20} \times 10^{13}$	Notes	Diameter $2r$
					$m\mu$
1	1	From S. E. $2 \times$ ultracentrifuged	350	Boundary very diffuse	50
2	9I	From 0.66 saturated $(\text{NH}_4)_2\text{SO}_4$ ppt. of B. M. E. $1 \times$ ultracentrifuged	205	$\Delta$ cm. per sec. fairly constant	38
3	9II	Sedimented from supernatant of Pellet 9I; $1 \times$ sedimented	125	Sharp boundary, early stages	30
4	9II	$1 \times$ sedimented	136	Boundary first well defined; disintegrates later on	31
5	2-7	Pellets from original S. E. pooled and sedimented	475	$\Delta$ cm. per sec. satisfactorily constant	58.4
6†	10	From 0.66 saturated $(\text{NH}_4)_2\text{SO}_4$ ppt. of S. E. $2 \times$ ultracentrifuged		Insufficient photographic contrast	
7	17	From 0.66 saturated $(\text{NH}_4)_2\text{SO}_4$ ppt. of S. E. $1 \times$ ultracentrifuged	548	$\Delta$ cm. per sec. satisfactorily constant after 5 min. sedimentation	62
8	16	" "		Very irregular boundary	
9	18	From 0.66 saturated $(\text{NH}_4)_2\text{SO}_4$ ppt. of S. E. $2 \times$ ultracentrifuged	110	Well defined boundary, measured directly	28
10	18	" "		Distance sedimented by boundary very short	
11	18	$2 \times$ ultracentrifuged	246	Photometered	42
Mean.....					42

S. E. = sonic extract; B. M. E. = ball mill extract.

\* Pellets 1, 9I, 9II, 2-7, 10 were photographed at a speed of 150 revolutions per second; Pellets 16, 17, and 18 at a speed of 120 revolutions per second. Pellet 1 was photographed in the short ultraviolet region; the remaining pellets were photographed in the long ultraviolet region.

† Strain 1685M.

*Electron Micrograph of Macromolecular Material*—A picture of macromolecular material from a single preparation (Pellet 20) by Dr. T. F. Anderson, taken with the RCA electron microscope, is shown in Fig. 4. In this are seen many dense spheroidal bodies whose diameters are given in Table III (Fig. 4 shows only a portion of the micrograph used). These diameters were computed from

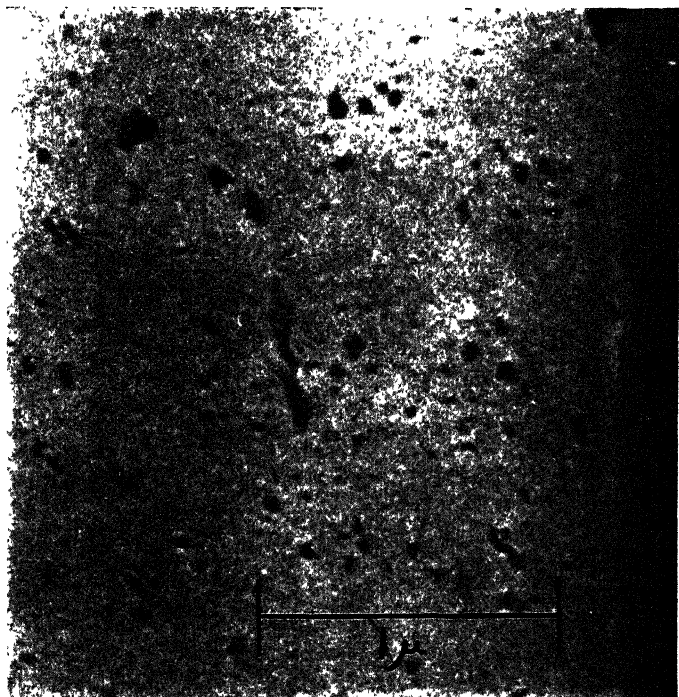


FIG. 4. Electron micrograph of heavy particles  $\times 40,000$  from *Streptococcus pyogenes*, Strain 1048M.

measurements of the particles, estimated to 0.1  $\mu$ , on the micrograph whose magnification is  $\times 40,000$ . The data in Table III indicate that there are two groups of particles of different sizes. One group ranges about a mean diameter of 21.2  $\mu$ , with a standard deviation of 6.6, and the other about a mean of 45.5  $\mu$ , with a standard deviation of 4.4. This range of diameters and mean diameters of 21.2 and 45.5  $\mu$  is in satisfactory agreement with the

corresponding values computed from the sedimentation data (see Table II).

In addition there is present an occasional irregularly shaped particle of lesser opacity which might conceivably be a fragment of the bacterial membrane<sup>5</sup> or other bacterial component.

*Electrophoresis*—Attempts were made to study various preparations of the ultracentrifugally purified macromolecular material in the electrophoresis apparatus of Tiselius (17), Toepler's schlieren method as well as Tiselius' light absorption method being employed for the optical analysis of the boundaries. Whereas the results

TABLE III  
*Diameters of Spheroidal Particles Computed from Electron  
Micrograph (Fig. 4)*

No. of particles	Diameter	No. of particles	Diameter
	<i>mμ</i>		<i>mμ</i>
9	7.5	2	35.0
8	10.0	10	37.5
12	12.5	6	40.0
15	15.0	4	42.5
19	17.5	3	45.0
22	20.0	1	47.5
10	22.5	6	50.0
29	25.0	1	52.5
6	27.5	1	55.0
16	30.0	1	75.0
1	32.5	1	115.0

obtained with the schlieren method were not satisfactory owing to the deep color and the strong Tyndall effect exhibited by the material, the light absorption method yielded adequate photographic records (see Fig. 5). In all experiments, the boundary of the colored material remained single and well defined throughout the run, indicating a high degree of electrochemical homogeneity. It might be mentioned that an analogous case of a material showing heterodispersity in the ultracentrifuge and homogeneity in the electrical field is presented by Stanley's earlier preparations of

<sup>5</sup> The bacterial membrane, free of inner protoplasm, has been shown to be a relatively transparent structure under the electron microscope (see (16)).

salt-treated tobacco mosaic virus (Eriksson-Quensel and Svedberg (18)). At pH 7.3, the *Streptococcus pyogenes* material (Pellet 20) migrates toward the anode with the high mobility,  $\mu = -17.5 \times 10^{-5} \text{ cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1}$  (corrected for water of 4°). The considerable charge density on the surface of the particles, as revealed by this electrochemical behavior, is probably related to the presence of a high phosphorus-containing material (see foot-note to Table I).

*Ultraviolet Light Absorption*—The ultraviolet absorption spectra of several ultracentrifugally purified preparations were kindly re-

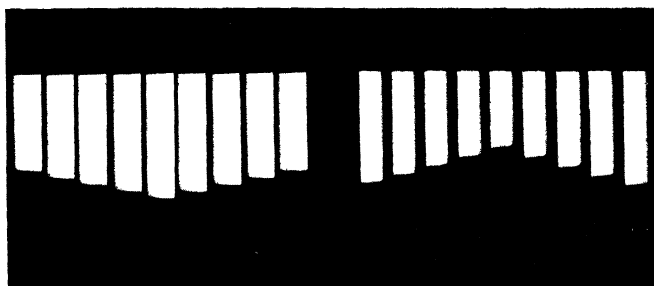


FIG. 5. Electrophoretic diagrams of macromolecular material from *Streptococcus pyogenes*, obtained with the light absorption method. Concentration, 16 mg. per cc.; solvent, 0.013 M phosphate buffer, pH 7.3; temperature 19°; simple cylindrical U-tube electrophoresis cell according to Tiselius; 22.5 volts, 0.27 milliamperes; potential gradient,  $F = 0.334 \text{ volt per cm.}$ ; light source, General Electric mercury arc lamp, Type H-2 (250 watts); no light filters; Eastman contrast lantern slide; 5 second exposures at intervals of 20 minutes (current reversed after 80 minutes); photography at magnification of 0.984 with spherically and chromatically corrected lens system,  $F = 730 \text{ mm.}$  (Perkins and Elmer Company).

corded by Dr. G. I. Lavin at The Rockefeller Institute for Medical Research and by Dr. R. Bowling Barnes at the laboratories of the American Cyanamid Company. The curve obtained by Dr. Barnes with an automatically recording spectrophotometer shows a slight absorption maximum near  $278 \text{ m}\mu$  and an inflection point near  $233 \text{ m}\mu$ . The absorption characteristics are not clearly brought out, owing possibly to the presence of lipids. The examination performed by Dr. Lavin with a hydrogen discharge tube as the light source and a small Hilger quartz spectrograph revealed the presence of narrow fine structure bands in the tryptophane and also probably in the tyrosine and phenylalanine region. cor-

responding to the 280 m $\mu$  spectral region. There is also an indication of absorption in the nucleic acid region (at 260 m $\mu$ ). The spectra were quite diffuse and suggested the presence of some kind of lipid material. Although the spectrographic data are far from being complete, it may be stated that they are compatible with the conclusions drawn from the chemical study of the macromolecular material.

#### SUMMARY

Pigmented, heavy particles, which may well be macromolecular in nature, have been isolated, by means of high speed centrifugation, from extracts prepared from two strains of *Streptococcus pyogenes*. The macromolecular substance is released from the bacterial cell into solution by disintegration with the aid of intense sonic vibration or grinding in a ball mill. A preliminary purification may be accomplished by precipitation of the material from such extracts with ammonium sulfate at 66 per cent saturation. Repeated differential centrifuging at high and low speeds in a Beams air-driven ultracentrifuge and the angle head of the International centrifuge, respectively, has proved an effective means of obtaining the macromolecular material in a purified state. A limit is set to this method of purification by the instability of the substance.

The purified macromolecular material contains lipid, nucleic acid, protein, carbohydrate, and a green pigment of unknown constitution. In the analytical ultracentrifuge, the purified material exhibits the behavior of a colloid of polydisperse character. If a spherical shape is assumed in view of the absence of double refraction of flow and a density value of 1.22 is taken, as determined by the falling drop method, a mean particle diameter of 42 m $\mu$  is calculated from the sedimentation rates observed by the light absorption method. Indeed, electron micrographs of one purified preparation show particles that correspond in size and shape to those deduced on the basis of the above evidence. In the Tiselius electrophoresis apparatus, the material migrates with a single boundary and with a mobility at 4° of  $17.5 \times 10^{-5}$  cm.<sup>2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> at pH 7.3.

The macromolecular material is antigenic. It leads to antibody formation when injected into rabbits. Furthermore, it appears to contain group-specific carbohydrate.

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# THE STORAGE OF THE MAJOR LIVER COMPONENTS; EMPHASIZING THE RELATIONSHIP OF GLYCOGEN TO WATER IN THE LIVER AND THE HYDRATION OF GLYCOGEN

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The work of a number of investigators, MacKay and Bergman on rabbits (1) and rats (2), Ling and Shen on ducks (3), Fenn (4) and Greisheimer and Goldsworthy (5) on rats, and Fenn and Haege (6) on cats, indicates that water storage in the liver accompanies glycogen deposition. Most of these workers have made the assumption that the absolute weight of the non-glycogen solids in the liver remains constant, while the glycogen and water vary. In an attempt to approach ideal conditions, MacKay and Bergman (2) sought "to alter solely the glycogen content of the liver." They fasted rats of the same age and sex for 48 hours, then weighed them, and fed pure glucose or fructose. Groups of the animals were killed at different intervals. The mean body weights of the animals within each experimental group are remarkably similar. Presumably the livers were comparable. If glycogen and water were the only variables, the content of non-glycogen solids (liver weight minus water and glycogen) should be the same in each group. Calculations from MacKay and Bergman's data are given in Table I. In the male rats fed glucose the non-glycogen solids increase as much as 32 per cent over the controls when the glycogen reaches its maximal value. The results were somewhat similar in female rats fed fructose. In these experiments the conditions were for the most part those existing during glycogenesis. Two possibilities suggest themselves; namely, that during glycogenesis either a glucose polymer is formed which is not determined by the Pflüger method, or else the process of glyco-



genesis serves as a stimulus for the deposition of non-carbohydrate material.

We have also given in Table I the values of the "apparent" ratios of glycogen to water. In calculating these ratios we have used the ratio of non-glycogen solids to water in Group 1 of each of the two sets as a control. This ratio is 1:2.3 for the males and 1:2.46 for the females. The former value has been corrected by subtracting from the liver water the amount associated with the glycogen, assuming that each gm. of the latter is associated with

TABLE I  
*Variation of Non-Glycogen Solids in Liver (Calculated from Data of MacKay and Bergman)*

Group No. (5 rats in each)	Body weight	Liver weight	"Absolute" weight of components per liver			Increase in non-glycogen solids	Apparent ratio, glycogen to water
			Water	Glycogen	Non-glycogen solids		
Males fed glucose							
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	
1	190	4.85	3.38	0.017	1.45	0	
2	189	6.41	4.38	0.245	1.78	22.8	1:1.1
3	186	7.34	5.03	0.473	1.84	26.9	1:1.6
4	187	7.94	5.46	0.553	1.92	32.4	1:1.8
Females fed fructose							
1	133	3.81	2.70	0.006	1.10		
2	138	4.86	3.44	0.228	1.13	2.7	1:2.9
3	136	6.03	4.24	0.395	1.40	27.2	1:2.0
4	138	6.53	4.58	0.492	1.46	32.6	1:2.0
5	138	6.79	4.83	0.574	1.39	26.4	1:2.4

2.7 gm. of water. The weight of the non-glycogen solids in each successive group has been multiplied by the respective factor and the product subtracted from the total water, yielding the weight of water associated with glycogen. Emphasis must be placed on the term "apparent." This is so because it was assumed that the same ratio holds for the new non-glycogen solids as for the original. This assumption is not justified; the ratio for these new solids is probably lower. If these increased solids are actually associated with less water than 2.3 or 2.46 gm. per gm. of solids, then these "apparent" ratios will be low as they are in the case of the male

rats. It is necessary to consider here the relationship of water to liver components other than glycogen as established by other workers. MacKay and Bergman, employing both their own data and those of Higgins *et al.* (7), have shown that each gm. of protein deposited is accompanied by 2 gm. of liver water, and that when fat accumulates in the liver there is no increase in water.

If the chemical nature of these increased solids remained constant as the amount of them increased, we should expect that the "apparent" ratios of glycogen to water would decrease. Instead, we find that these ratios for the male rats increase. Evidently the nature of these solids changes. We are investigating this problem further.

If, in our effort to establish the relationship of water and glycogen, we select as a criterion of comparability the constancy of the non-glycogen solids between the control and the experimental animals, then we find only Groups 1 and 2 of the females to fill these requirements. In this instance the ratio of 1 gm. of glycogen to 2.9 gm. of water, we shall show later, agrees fairly well with our own values.

In spite of the excellent data of MacKay and Bergman, it appears that their method for establishing the relationship between glycogen deposition and water storage involves the use of unjustifiable assumptions. In the work here reported we have investigated the variation of water, glycogen, and non-glycogen solids under certain dietary and physical conditions, including normal feeding.

### Methods

In all of our experiments 100 day-old male rats were used.

The method of glycogen determination was that of Blatherwick *et al.* (8). The glycogen values were obtained by multiplying the glucose recovered after hydrolysis by 0.927.

The agar-fed rats ate about 5 gm. of a mixture of 100 gm. of agar, 4 gm. of mineral oil, and 1 gm. of Liebig's beef extract. The rats on limited feeding in Experiment 2 (Table II) received 50 per cent and in Experiment 9 (Table IV) about 40 per cent of the normal intake of stock diet which is relatively high in carbohydrate. Following the 36 hour fasting period the rats in Experiments 2, 3, 9, and 10 were fed 12 hours prior to the end of the experiment.

Lipids were determined by a modified Bloor method. Five

TABLE II

Relation of Non-Glycogen Solids and Water Associated with Glycogen (Unadjusted Data)

Experiment No.	Dietary procedure	N o. of rats	Average final weight		Average per cent in liver		Average weight of component per liver				"Appar- ent" ratio, glyco- gen to water
			Whole animal	Wet liver	Water	Glycogen	Dry solids free of glycogen	Glycogen	Total water	Water asso- ciated with glyco- gen	
1	Fed agar 24 hrs.	19	237 ± 7.3	6.1 ± 0.067	71.5 ± 0.12	0.0	1.77 ± 0.059	0.00	4.31 ± 0.15		
2	Fasted 36 hrs., then fed 50% normal intake	8	226 ± 10.4	7.6 ± 0.37	71.3 ± 0.18	5.98 ± 0.20	1.74 ± 0.085	0.450 ± 0.028	5.44 ± 0.28	1.21	
3	Fasted 36 hrs., then full fed	14	256 ± 6.1	9.5 ± 0.27	70.1 ± 0.16	8.34 ± 0.12	2.06 ± 0.062	0.784 ± 0.003	6.71 ± 0.18	1.72	
4	Prefed stock diet, then fasted 24 hrs.*	8	237 ± 8.5	7.5 ± 0.23	70.7 ± 0.13	1.93 ± 0.21	2.05 ± 0.062	0.144 ± 0.012	5.26 ± 0.16	0.32	
5	Fed protein 1 wk.	8	198 ± 4.4	6.9 ± 0.29	71.4 ± 0.24	2.33 ± 0.16	1.81 ± 0.070	0.162 ± 0.010	4.91 ± 0.17	0.51	
6	Prefed protein, then fasted 24 hrs.	8	237 ± 12.2	6.9 ± 0.26	70.2 ± 0.32	0.96 ± 0.066	2.00 ± 0.065	0.066 ± 0.006	4.87 ± 0.17	0.00	
7	" "	8	226 ± 7.0	8.16 ± 0.33	70.0 ± 0.30	3.37 ± 0.28	2.16 ± 0.079	0.279 ± 0.033	5.72 ± 0.27	0.47	

\* In Experiments 4 and 7 the rats were fasted under 0.5 atmosphere of pressure. Except in these two experiments water was accessible during the fasting period.

extractions with 100 cc. portions of 95 per cent ethanol were made of 30 gm. lots of the frozen crushed tissue. This sufficed to remove practically all of the extractable material, as was shown by a subsequent Soxhlet extraction with dry ethyl ether.

The ash and nitrogen determinations were made only upon the dry fat-free solids. Nitrogen determinations were made by a semi-micro-Kjeldahl method. The values should represent only protein, since most of the non-protein nitrogen compounds presumably were removed by the alcoholic extraction. Water determinations were made by drying portions of frozen crushed tissue to constant weight on a steam bath.

*Concerning Precision*—All values in Table II are arithmetic means, and with each is given the deviation of the mean as  $\epsilon_M$ .  $\epsilon = \sqrt{\sum d^2/N - 1}$ ,  $\epsilon_M = \epsilon/\sqrt{N}$ .

The raw data show a fairly even distribution of light and heavy rats. This is reflected in the reasonably uniform  $\epsilon$ .

The precision of the analyses of glycogen and of water is of high order. These precisions determine the "apparent" ratio of glycogen to water, since it can be shown that these ratios are independent of other measurements.

In marked contrast is the low precision of the body and liver weights. These values could be improved by selecting the animals, as did MacKay and Bergman, to fall within narrow tolerance limits of the mean body weight.

Of somewhat higher order than that of the body and liver weights is the precision of the factors used in adjusting the final to the initial body weights.

#### EXPERIMENTAL

Table II contains all of the raw data from seven independent experiments. The results of the analyses of the components are given both as percentage and "absolute" values. Experiment 1 serves to establish the ratio of non-glycogen solids to water (1:2.43).

In most of our experiments litter mates were divided among the experimental groups. However, the mean body weights are not the same in all of the groups. Some adjustment of values is necessary. In Table II is given only the final body weight at the conclusion of the experimental diet. It was not until after the

calculations were attempted that we realized the desirability of adjusting to the initial rather than to the final body weight. Therefore, we repeated all except Experiment 7, but determined only the effect of the dietary procedure upon the body weight. The same experimental conditions were maintained throughout. Using these new data, we adjusted the liver weight in Table II to a uniform initial body weight of 256 gm. which is both the initial and the final weight of the full fed (stock diet) rats in Experiment 3.

TABLE III  
*Adjustment Factors and Adjusted Liver Weights*

Experiment No.	Dietary procedure	Loss of initial body weight due to diet	Final body weight		Adjusted liver weight	Glycogen-free solids in liver	"Apparent" ratio, glycogen to water
			Calculated	Observed			
		<i>per cent</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		
1	Fed agar for 24 hrs.	8.7	234	237	6.0	1.71	
2	Fasted 36 hrs., then fed 50% normal intake	8.3	235	226	7.9	1.80	1:2.7
3	Fasted 36 hrs., then full fed	0		256		2.06	1:2.1
4	Prefed stock diet, then fasted 24 hrs.	10.9	228	237	7.22	1.98	1:2.2
5	Fed protein 1 wk.	6.0	241	198	8.40	2.21	1:3.0
6	Prefed protein, then fasted 24 hrs.	13.2	222	237	6.46	1.86	?

In these rats the wet stomach contents alone account for an average of 17 gm. of the final body weight.

#### DISCUSSION

In Table III are given the adjusted liver weights and the data from which they were calculated. Table III shows the degree of comparability between the animals in the different groups, the index of this being the constancy of the non-glycogen solids. Experiments 1 and 2 come close to meeting these requirements. Therefore, the ratio of 1 gm. of glycogen to 2.7 gm. of water is as nearly correct as can be obtained from our data and by our method.

The most important conclusion to be drawn from Tables II and III is that the "apparent" ratio of glycogen to water varies with a change in the content of non-glycogen solids of the liver, generally decreasing as the latter increases. However, the results in Experiments 5 and 6 are an exception to this. The rats fed the commercial dry meat scrap powder in these experiments yield results different from those fed the stock diet. Thus, in Experiment 6 although the liver contains nearly 1 per cent of glycogen there appears to be neither any accompanying water nor change in the non-glycogen solids.

We have used the agar diet (developed by Guest) for our controls because it rendered unnecessary the correction of their liver solids for glycogen. Our choice was also influenced by the possibility that these controls may be more nearly comparable to the rats fed a nutrient diet, because their gut activity is no doubt greater than that of the fasted rats.

#### *Further Experiments*

It was decided to repeat Experiments 1, 2, and 3 to determine the content of the major liver components. Accordingly, three entirely independent experiments, Nos. 8, 9, and 10, were run. In these the livers were frozen, weighed separately, crushed, and mixed. The results of the analyses are given in Table IV. The values only for the final body weights are given. The initial mean body weight in Experiment 8 was known to be 270 gm. For this reason the liver weight in Experiment 10 was adjusted so that all three groups correspond to the same initial body weight of 270 gm.

The "absolute" values of the separate liver components indicate that glycogen is the only important variable in the rats on limited feeding in Experiment 9. When the fat content in Experiments 8 and 10 is compared, it will be seen that it has increased by 0.21 gm. in Experiment 10. This increase of fat is large enough to account for the increase in non-glycogen solids, as can be seen by subtracting this weight from 2.05. The difference, 1.84, agrees very well with the values in Experiments 8 and 9. If we recalculate the ratio of glycogen to water using this "equi-fat" value for the non-glycogen solids of 1.84, a ratio of 1:2.6 is obtained. The glycogen in the full fed rats must be associated with the same weight of water as in the rats on limited feeding in Experiment 9.

Furthermore, if this excess of 0.21 gm. of fat is subtracted from the liver weight in Experiment 10 the difference, 9.52, compared to 6.85 gm. of water, yields by calculation a corrected percentage of water equal to 71.8, which also agrees with that of the controls. The increase in liver fat is reflected both in a low value for the apparent ratio of glycogen to water and in a relatively low percentage of liver water. This observation is consistent with the fact that the deposition of fat is not accompanied by an increase

TABLE IV  
*Variation of Major Liver Components*

Experiment No.	Dietary procedure	No. of rats	Average final weight		Average per cent in wet liver				
			Whole animal	Wet liver	Water	Protein (N $\times$ 6.25)	Lipids	Glycogen	Ash
			gm.	gm.					
8	Fed agar	6	249	6.4	71.6	22.0	4.44	0.1	0.74
9	Fed limited stock diet	5	248	7.3	72.0	19.0	4.46	3.0	0.63
10	Full fed stock diet	5	286	10.3	70.4	15.3	5.11	8.5	0.83

	Average weight		Average weight of components per liver						"Apparent" ratio, glycogen to water
	Wet liver*	Dry solids free of glycogen	Protein	Lipids	Ash	Glycogen	Total water	Water associated with glycogen	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	
8	6.4	1.81	1.41	0.286	0.048	0.00	4.63		
9	7.3	1.83	1.39	0.325	0.046	0.212	5.26	0.59	1:2.7
10	9.73	2.05	1.49	0.496	0.080	0.826	6.84	1.58	1:1.9

\* These values correspond to an initial body weight of 270 gm.

in liver water. The difference between the percentages of water in Experiments 1 and 3 (Table II), equal to 1.4, is statistically significant. In all of the experiments in Tables I to IV there is evidence of a correlation between a low "apparent" ratio of glycogen to water and a relatively low percentage of liver water. There is a close resemblance between our data and those of MacKay and Bergman. If the increased non-glycogen solids in certain of their rats consisted largely of fat, this would explain the very low "apparent" ratios of glycogen to water we have calculated.

We are convinced that in a quantitative study of liver storage it is essential to control as many of the biological factors as possible before valid conclusions can be drawn. In view of our findings the ratios of water to glycogen and protein as established by Fenn and Fenn and Haeger are probably apparent rather than real, since their method of calculation demands that the relationship of water to the individual non-glycogen tissue components is the same whether these components are organized as an essential part of the cell or enter as storage material. Their method offers no way of determining the extent of such storage. On the other hand, their findings as to the constancy of the relationships of water and of glycogen to chlorides and potassium, etc., are very likely independent of the absolute weight of the non-glycogen solids. These latter findings indicate that the water associated with glycogen cannot be regarded as "bound" water, because if it were "bound" one would expect a departure from isosmotic conditions.

#### *Hydration of Glycogen*

The following is an attempt to explain the fact of water storage in glycogen deposition. We shall assume that the water here referred to is actually associated with the glycogen. It is not likely that this water plays a rôle in the maintenance of isosmosis, because the osmotic pressure of 1 gm. of glycogen in 2.7 gm. of water is too small compared to that of the tissue salts. In checking this point values for the molecular weight of glycogen between 1 and 2 million were used. This range has been observed by McBride and Beckmann in some unpublished ultracentrifuge studies.

There is a possibility that this water may be held in hydrate formation by the glycogen. The ratio of the two fractions 2.7/18 to 1/162 corresponds to 24 water molecules (six tetrahydrol units) per glucose unit. The existence of tetrahydrol units in water, as shown by Bernal and Fowler (9) and others, and the presence of three free hydroxyl groups per glucose unit suggest that 12 water molecules may associate per glucose unit. On the other hand, by the use of the Stewart-Hirschfelder atomic models one can show that 24 water molecules are probably slightly more than enough to form a monomolecular film around each glucose unit.



## SUMMARY

1. The experimental data of MacKay and Bergman on rats were recalculated and interpreted to indicate that during glycogenesis there is an increase of as much as 32 per cent of unidentified non-glycogen solids in the liver.

2. Evidence is offered to show that the "apparent" ratio of glycogen to water varies with an increase in the content of non-glycogen solids of the liver. The results are different for rats fed protein than for those fed a high carbohydrate diet.

3. In fasted rats prefed with protein there appears to be no water storage accompanying low glycogen concentrations.

4. When there is no change in the non-glycogen solids of the liver, each gm. of glycogen deposited is associated with approximately 2.7 gm. of water.

5. There is evidence of a correlation between a relatively low percentage of liver water and a low "apparent" ratio of glycogen to water. In rats fed a stock diet it can be shown that this correlation is accounted for by a deposition of liver fat during glycogenesis.

6. The ratio of glycogen to water of 1:2.7 corresponds to 24 water molecules (or six tetrahydrol units) per glucose unit in glycogen. Theoretical considerations lead one to expect a possible association of 12 water molecules (three tetrahydrol units) per glucose unit in a higher hydrate.

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## PHOSPHOLIPID METABOLISM IN DENERVATED MUSCLES\*

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Several studies indicate that the more active the skeletal muscle the higher its phospholipid content (6-8). Phospholipids are diminished in muscular atrophy and dystrophy (13, 6), except in the dystrophy of vitamin E-deficient rabbits which show an increase (23). No change (16) or a decrease (10) in the phospholipids, calculated as per cent of dry and defatted tissue, has been described in muscles after denervation.

In the present study radioactive phosphorus has been used as an indicator to investigate the phospholipid metabolism in denervated muscles of rats<sup>1</sup> and cats. Since the completion of the experiments, data on the radioactivity of phospholipids in denervated and intact muscles of rats injected with labeled phosphate have been published by others, their conclusions being similar to our own (15). In most of our experiments comparative estimations of both the total and radioactive phosphorus have been made on muscle lipids as well as on those of plasma and liver and on the inorganic phosphate of plasma. Data after introduction of labeled phosphate have been compared with those obtained after administration of radioactive phospholipids. Therefore it is presumed that the results reported below, besides giving further information on the phenomena, provide perhaps a better basis for their discussion and interpretation.

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<sup>1</sup> The experiments on rats were made in 1938 in the Departments of Physiology and Physics of the University of Palermo, Italy.

## EXPERIMENTAL

On four rats and five cats the sciatic and femoral nerves of the right leg were cut aseptically, the operation resulting in the paralysis of all the muscles of the leg and several of those of the thigh. From the 3rd to the 6th day after denervation, the rats were injected daily with an isotonic solution of sodium phosphate (pH 7.4) containing the radioactive isotope  $P^{32}$ , and killed by decapitation 24 hours after the last injection. On Cats I to IV the total dose of radioactive phosphate was injected at one time. Cat V was injected intravenously with an emulsion of radioactive phospholipids extracted from the liver of another cat which had previously received a large dose of labeled phosphate. The phospholipid emulsion was prepared as described by Haven and Bale (17) with minor modifications. After various intervals, the cats were bled from the carotid artery and the blood collected over sodium fluoride.

Two solutions with different phosphorus content and different radioactivity were used for the rats and the cats.

*Procedures*

*Analytical Methods*—Samples weighing from 6 to 18 gm. were removed from the paralyzed muscles and from the corresponding muscles of the intact leg, dehydrated with alcohol at room temperature, ground in a mortar, and extracted with boiling alcohol in a continuous extraction apparatus of the Kumagawa-Suto type. After evaporation of the alcohol under reduced pressure, the extracts were redissolved in chloroform and filtered through asbestos.

Because of the presence in muscles of large amounts of phosphorus-containing substances other than the phospholipids, in several experiments the muscle phospholipids have been isolated from the chloroform extracts by three subsequent precipitations with acetone and  $MgCl_2$  and further purified by a procedure essentially similar to that of MacLean (21) as adapted to the phospholipids in animal tissues by Le Breton (20) (Table II).

For the extraction of lipids from liver samples (3 to 5 gm.) a semimicroprocedure previously described (2) has been used. Plasma lipids have been extracted according to Folch and Van Slyke (14) and purified by chloroform.

Total lipids have been evaluated from the weight of the chloroform extracts. The phosphorus content of these extracts, or of the purified phospholipids, was determined by Tisdall's method (27) after digestion with sulfuric and nitric acids. Inorganic phosphates were directly precipitated as strychnine phosphomolybdate in the trichloroacetic acid filtrates of the plasma. Muscle "proteins" have been estimated either by weighing the dry residue from the alcohol extraction or by determining the total N in the moist tissue. The two methods of calculation gave practically the same figures.<sup>2</sup>

*Radioactivity Measurements*—In the experiments on rats, after incineration of the lipid extracts the radioactivity of the phosphomolybdic precipitates was measured with an ionization chamber; details of the procedure have been given elsewhere (1, 2).

In the experiments on cats, an aliquot of the chloroform solutions was shaken with pulverized sodium phosphate and filtered into a small aluminum dish. The lipid content of the sample was brought to a definite value by adding a suitable amount of a chloroform solution of egg yolk lipids or phospholipids. After evaporation of the solvent, the radioactivity was measured with a Geiger-Müller counter and compared with the radioactivity of standard samples, determined simultaneously under identical conditions. The standards contained the same total weight of egg lipids and various amounts of the solution of labeled phosphate used in the experiments.

The radioactivity of the plasma inorganic phosphate was compared with that of another series of standards prepared by precipitating with the strychnomolybdic reagent an aliquot of the original solution of radioactive phosphorus to which suitable amounts of inactive inorganic phosphate had been added.

The radioactivity has been expressed in relative radioactive units (r.r.u.), the total dose injected in the animal being considered equal to  $10^4$  r.r.u. As in previous experiments (1-3), the ratio between the radioactivity of the sample (in r.r.u.) and

<sup>2</sup> The values of muscle "proteins" are mere approximations. Most of, but possibly not all, the extractives have been removed from the tissue by double extraction with cold aqueous alcohol and with boiling strong alcohol. On the other hand, in order to correct for the presence of nitrogenous extractives, the figures for total N have been multiplied by 6 (instead of 6.25); but obviously this correction is arbitrary.

its content of phosphorus (in mg.) has been called "specific activity."

*Approximation of Results*—The statistical error of the radioactivity measurements was generally between 4 and 6 per cent, while the analytical error of the phosphorus analyses, as estimated by duplicate determinations, was below 3 per cent.

Comparative estimations of phosphorus and radioactivity have been made on the lipids extracted from the two legs of one normal rat and one normal cat previously injected with radioactive phosphate. The following values (in per cent of muscle "proteins") were obtained: in the rat P 1.83 and 1.88 mg., r.r.u.  $283 \pm 15$  and  $299 \pm 13$ , specific activity 154.5 and 159.0; in the cat P 2.10 and 2.00 mg., r.r.u.  $14 \pm 0.6$  and  $13.5 \pm 0.7$ , specific activity 6.67 and 6.75.

#### RESULTS AND DISCUSSION

Data of our radioactivity measurements on the muscles of rats coincide with those obtained from a large number of animals by Friedlander *et al.* (15); therefore only the results of one of our four experiments on rats are reported as an example in Table I. In our experiments on cats the radioactivity is also increased after denervation. The increase is apparent in the total lipid extracts as well as in the fully purified phospholipids<sup>3</sup> (Table II) and is mostly accompanied by a less conspicuous increase in total phospholipids; consequently the specific activity is higher in the denervated muscles.

In agreement with some previous reports ((4, 10, 12, 16); see also (9, 28)), total lipids appear to increase after denervation, whereas muscle "proteins" are often slightly decreased. Therefore, if our results are calculated per gm. of "proteins," the difference between the content of phospholipids in the denervated and in the intact muscles is even greater.

<sup>3</sup> The effectiveness of the purification is substantiated by the fact that the ratios between the fatty acids, determined acidimetrically in the saponified acetonetic precipitates, and the phosphorus of the purified phospholipids have been in three determinations 18.1, 17.6, and 18.5; that is, almost the theoretical value for pure lecithins and cephalins (18.06, assuming a mean molecular weight of 280 for their fatty acids).

TABLE I

*Results of Chemical and Radioactive Determinations on Plasma, Liver, and Muscles of Rats and Cats Injected with Labeled Phosphate or Phospholipids*

Values per 100 gm. of moist tissue or 100 cc. of plasma.

Animal No.		Days after denervation	Days after administration of $P^{32}$	Lipid extracts from				Plasma inorganic phosphates
				Intact muscles	Denervated muscles	Plasma	Liver	
Rat C	"Proteins," gm.	7	*	21.7	20.9			
	P, mg.			39.4	41.4		131.1	
	R.r.u.			716	1195		6672	
	Specific activity			18.2	28.8		50.9	
Cat I	"Proteins," gm.	7	2	19.3	19.1			
	Total lipids, gm.			2.54	3.18	0.426	6.64	
	P, mg.			37.1	42.5	6.52	148.2	5.95
	R.r.u.			50.5	72.3	49.4	1455	68.7
" II	Specific activity	10	4	1.36	1.70	7.56	9.82	11.5
	"Proteins," gm.			19.3	17.7			
	Total lipids, gm.			3.43	4.16	0.495	6.74	
	P, mg.			36.2	40.7	6.47	114.5	6.16
" III	R.r.u.	10	6	37.7	93.2	27.5	595	38.8
	Specific activity			1.04	2.29	4.25	5.20	6.30
	"Proteins," gm.			19.1	18.8			
	Total lipids, gm.			2.00	2.82	0.358	6.45	
" IV	P, mg.	14	8	36.3	41.6	5.11	148.2	5.95
	R.r.u.			65.8	97.8	15.4	488	25.7
	Specific activity			1.81	2.35	3.02	3.30	4.34
	"Proteins," gm.			17.5	16.8			
" V†	Total lipids, gm.	11	6	2.26	3.95	0.603	6.37	
	P, mg.			35.3	43.6	5.86	125.8	5.35
	R.r.u.			89.0	110.0	14.6	324.4	14.2
	Specific activity			2.52	2.52	2.49	2.58	2.65
	"Proteins," gm.			18.5	18.3			
	Total lipids, gm.			3.00	3.84	0.470	6.41	
	P, mg.			43.1	48.5	5.43	151.2	5.04
	R.r.u.			27.4	73.0	31.5	1003	8.9
	Specific activity			0.64	1.51	5.81	7.24	1.76

\* Injected daily with the solution of radioactive phosphate from the 4th to 6th day after denervation.

† Injected with the emulsion of radioactive liver phospholipids.

In cats injected with labeled phosphate (except in Cat IV<sup>4</sup>) the specific activity values show a gradient in the following order: plasma inorganic phosphate, liver, plasma, denervated and intact muscle lipids. The same gradient for the specific activities of the phospholipids was obtained in Cat V after introduction of the emulsion of radioactive phospholipids. These findings are in line with the idea that phospholipids synthesized from the plasma

TABLE II

*Phosphorus, Radioactivity, and Specific Activity in Various Stages of Purification of Muscle Phospholipids*

The values are given in mg. of phosphorus and relative radioactive units per gm. of muscle "proteins."

Animal No.		Intact muscles			Denervated muscles		
		Total chloroform extract	Acetone + MgCl <sub>2</sub> ppt.	Purified phospholipids	Total chloroform extract	Acetone + MgCl <sub>2</sub> ppt.	Purified phospholipids
Rat C	P, mg.	1.82	1.70		1.98	1.92	
	R.r.u.	33.0	29.2		57.2	52.5	
	Specific activity	18.2	17.2		28.8	27.3	
Cat I	P, mg.	1.91	1.83		2.22	2.10	
	R.r.u.	2.62	2.36		3.78	3.75	
	Specific activity	1.36	1.29		1.70	1.78	
" II	P, mg.	1.88	1.81	1.45	2.30	2.11	1.93
	R.r.u.	1.95	1.86	1.39	5.26	4.86	3.66
	Specific activity	1.04	1.03	0.96	2.29	2.30	1.90
" III	P, mg.	1.90		1.66	2.22		1.80
	R.r.u.	3.45		2.49	5.20		3.61
	Specific activity	1.81		1.50	2.34		2.00
" IV	P, mg.	2.02		1.55	2.60		2.03
	R.r.u.	5.09		3.69	6.55		5.07
	Specific activity	2.52		2.38	2.52		2.50

phosphate in the liver (probably also in the intestinal mucosa) are carried in the plasma and deposited in the muscles.<sup>5</sup>

<sup>4</sup> In this animal, killed at the longest interval after the administration of P<sup>32</sup>, the specific activity of the plasma phosphate was practically the same as that of the phospholipids in liver, plasma, and muscles, as would logically be expected.

<sup>5</sup> For previous data obtained by using radioactive phosphorus in investigations on phospholipid metabolism and for a discussion on their significance, see, besides our earlier papers (1-3), the recent review by Hevesy (18).

If this interpretation is correct, the difference between the phospholipid contents of denervated and intact muscles may result from an increased deposition in the denervated muscles or from an increased destruction (or mobilization<sup>6</sup>) in the intact muscles or from both. As the specific activity is also increased in the denervated muscles, the first hypothesis seems more probable, unless it is assumed that the newly formed phospholipids are preferentially used (or mobilized) in the intact muscle. Experiments on this point are in progress in this laboratory.

Aside from any speculation on its mechanism of production, the increased concentration of phospholipids in denervated muscles apparently disagrees with Bloor's concept of a parallelism between the phospholipid content of skeletal muscles and their activity (6-8). In this respect the following points should be kept in mind. (a) Denervated muscles are far from inactive. Fibrillation is already apparent on the 3rd day after denervation in the rat's gastrocnemius (19) and on the 5th day in the cat's (25). As a consequence of the fibrillation, an analogy between denervated and fatigued muscle has even been suggested (24). (b) If the distinction between different classes of phospholipids with different physiological rôles ("non-metabolic" and "metabolic" (26) or "protoplasmic" and "lipometabolic phospholipids" (3)) is accepted, differences in the amount of the compounds of the first class should account for the different contents of phospholipids in muscles showing a different activity, whereas the increase of phospholipids in the denervated muscles would concern lipometabolic phospholipids only. Possibly these phospholipids acting as a transport form for fatty acids (as well as the other lipids which are also increased in the denervated muscles; see the figures for total lipids in Table I) are mainly present in the interstitial tissue rather than in the specific muscle cells.

As for the different behavior of phospholipids in muscular atrophy and dystrophy from various causes (13, 6, 23), the following possibility might be suggested as a working hypothesis: muscle alterations which are primarily dependent upon the loss of nerve control would be accompanied by an increased deposition of "lipometabolic phospholipids" and other lipids, whereas atrophies of purely mus-

<sup>6</sup> In this connection it should be noted that mobilization of fat from fat deposits is impaired by denervation, whereas deposition is not (22, 5, 11).



cular origin are perhaps followed by a decrease of "protoplasmic phospholipids".

#### SUMMARY

Rats and cats, in which the femoral and sciatic nerves of one leg had previously been cut, were injected with radioactive phosphate.

In the denervated muscles the total lipids, the newly formed phospholipids, and, to a less extent, the total phospholipids are increased. The increase is also apparent in determinations on fully purified phospholipids and is even greater, if values are calculated per gm. of dry muscle proteins.

The gradient of the specific activity values (ratios of radioactivity to the weight of phosphorus) suggests that phospholipids synthesized by the liver from plasma phosphate are carried in the plasma to the muscles, larger amounts being probably deposited in the denervated muscles.

Similar results have been obtained after introduction of an emulsion of labeled liver phospholipids.

The author wishes to thank Professor E. O. Lawrence and the members of the staff of the Radiation Laboratory of the University of California, who by the generous gift of the radioactive phosphorus made possible this investigation. He is also greatly indebted to Dr. E. Segrè, former professor of Physics at the University of Palermo, for the radioactivity measurements in some of the experiments on rats; to Dr. H. S. Wells of this Medical School for helpful advice and assistance; and to Mrs. Bianca Artom for most of the radioactive readings in the experiments on cats.

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## STUDIES IN AMINO ACID METABOLISM

### VI. THE METABOLISM OF *DL*-VALINE AND *DL*-ISOVALINE IN THE NORMAL RAT\*

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(Received for publication, January 14, 1941)

The indispensability of valine in the diet of the rat has been demonstrated (1). However, experiments designed to study the disposal of this amino acid when fed in large amounts have failed to yield satisfactory information as to its fate. Thus, Embden, Salomon, and Schmidt (2) by using liver perfusion methods could find no acetone bodies in the perfusing fluid on the addition of valine. Dakin (3) in three phlorhizin experiments was unable to demonstrate the production of an appreciable amount of glucose after feeding valine. He concluded that this amino acid did not give rise to sugar. He did make one rather significant statement: "The excretion of acetoacetic acid was distinctly lowered on giving valine." Chase and Lewis (4) in a study concerned primarily with rates of absorption failed to find any increase in liver glycogen 6 hours after feeding valine or isovaline ( $\alpha$ -amino- $\alpha$ -methylbutyric acid).

The work here reported is a study of the glycogenic property of the valines and the ketolytic property of *DL*-valine.

#### EXPERIMENTAL

The experimental procedures used in this study are the same as those employed in earlier studies concerned with the metabolism of the amino acids (5). These involve the feeding of the substrate and sacrificing the animal after varying time intervals for the

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was allowed to develop spontaneously, qualitatively the results are the same. After *dl*-valine was fed, the total acetone body excretion was lower than the corresponding control group. Table II lists the results of the exogenous type of ketonuria, in which

TABLE II

*Ketonuria in Female Rats Fed 15 Gm. of Sodium Butyrate (Calculated As Acetone) per Sq.m. of Body Surface per Day*

One group received in addition 12.50 gm. of *dl*-valine per unit area per day, fed in 7 per cent aqueous solution. The control group received sodium chloride solution. The animals were fasted the preceding 48 hours; five animals in each group for each day.

Material fed	Daily excretion of acetone bodies		
	1st day	2nd day	3rd day
	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>
<i>dl</i> -Valine. . . . .	6.90	8.38	7.69
Control . . . . .	14.48	12.84	13.73

All results are statistically significant by the *t* test of Fisher.

TABLE III

*Effect of Feeding 12.50 Gm. of dl-Valine per Sq.m. of Body Surface per Day on Ketonuria Developed from Endogenous Stores in Female Rat*

For the first 2 days only, 7.5 per cent sodium chloride solution was fed to each group. *dl*-Valine was fed in 7 per cent aqueous solution; eight animals in each group for all days.

Material fed	Daily total acetone body excretion				
	1st day	2nd day	3rd day	4th day	5th day
	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>	<i>gm. per sq.m.</i>
<i>dl</i> -Valine. . . . .	0.09	0.68	0.31	0.53	0.20
Control . . . . .	0.10	0.70	1.24	1.89	2.29

The 3rd, 4th, and 5th days are highly significant statistically by the *t* test of Fisher, when compared to the controls.

sodium butyrate was fed, while Table III contains the data from the endogenous type of experiments.

Since there was considerable variation between the individual animals, the data were subjected to statistical evaluation by the *t* test of Fisher (10). The foot-note below each table shows significance. In all cases a high degree of significance was observed.

## DISCUSSION

Although the values for liver glycogen after *dl*-valine feeding are not great, they are significant. If one considers the increase over the control, the values are of about the same order as for glycine (11), which is usually considered as a sugar-forming amino acid. MacKay *et al.* (12) have lately reported that glycine, after a latent period, is quite good as a glycogenic agent. Some 14 hours after a single dose of this amino acid was fed, the liver glycogen rose to a rather high level. Similar experiments, although not reported in detail, were carried out after a single dose of *dl*-valine. The experimental periods were of 16, 20, and 24 hours. Under these conditions insignificant amounts of liver glycogen were formed, thus indicating that valine does not have a latent period which characterizes glycine.

These results are in sharp contrast to those found after *dl*-isovaline is fed. In these experiments no glycogen was found in any of the groups.

The effect of feeding *dl*-valine to animals suffering from a ketosis is what one would predict from the glycogen studies; namely, a lowering of the excretion of the acetone bodies. This is shown both by the experiments in which a high ketonuria was produced by the feeding of sodium butyrate and in those in which an acetoneuria resulted from endogenous sources. In the latter study it is believed that this condition is traceable to the inclusion of fresh liver in the diet of our stock animals. McHenry and Gavin (13) have reported that a fraction prepared from fresh liver can cause a deposition of large amounts of fat in the liver. This is possibly the cause of the rather high level of acetone body excretion. In the experiments in which the sodium butyrate was superimposed on the already existing ketosis, a very high ketonuria resulted. In fact, we were recovering in the urine of the control animals almost as much material as we were feeding. The animals were receiving 15 gm. of sodium butyrate (calculated as acetone) and excreting from 12.84 to 14.48 gm. of total acetone bodies per sq.m. of surface area per day. However, regardless of the height of the ketonuria, feeding valine did cause a distinct lowering of the acetone body excretion.

In the experiments reported in Table III in which the ketonuria was developed from endogenous stores, during the first 2 days of

the experiment, in order to insure that we were dealing with comparable groups of animals, only a solution of sodium chloride was fed. This was given to insure a large urine volume. The animals were arranged so that the level of ketonuria was as nearly comparable between the two groups as possible. Again a distinct lowering of the acetoneuria followed the feeding of the valine on the last 3 days of the experiment.

In all of the various techniques used each demonstrates that valine may be classified as a sugar-forming amino acid, although this property is not great. We have no information as to whether only one isomer has the ability to give rise to glycogen or whether both forms may be active. One must await the resolution of the racemic mixture to answer this question.

#### SUMMARY

1. *dl*-Valine, when fed to rats, has been shown to give rise to a small but significant amount of liver glycogen.
2. *dl*-Isovaline is devoid of any glycogenic properties.
3. When *dl*-valine is fed to a rat suffering from a ketosis, the excretion of acetone bodies is markedly decreased. This is true both in a ketonuria arising from endogenous stores and when the feeding of sodium butyrate increases the already existing acidosis.

We are indebted to Dr. W. J. Kirkham of the Mathematics Department for his generous assistance in the statistical analysis of the data reported in this paper.

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## LETTERS TO THE EDITORS

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### THE SIGNIFICANCE OF A PATHWAY OF CARBOHYDRATE BREAKDOWN NOT INVOLVING GLYCOLYSIS\*

Sirs:

There is ample evidence from *in vitro* experimentation for at least two paths of carbohydrate breakdown in cells of bacteria, yeast, Amphibia, and mammals. One path (Reaction I) involving glucose phosphate  $\rightarrow$  triose phosphate  $\rightarrow$  phosphoglyceric acid  $\rightarrow$  pyruvic acid  $\rightarrow$  CO<sub>2</sub> and H<sub>2</sub>O is inhibited by fluoride at phosphoglyceric acid and by iodoacetate at triose phosphate. It has been demonstrated that the oxidation of glucose may take place despite the presence of these inhibitors. We wish to present *in vivo* observations on a mammal (rat) which corroborate the conception of multiple paths for the oxidation of glucose.

Young rats (1 day old) are able to live in an atmosphere of nitrogen for approximately 50 minutes. The energy permitting this survival period is provided in large part by the anaerobic cleavage of carbohydrate, for when such rats are injected with iodoacetate the length of survival in nitrogen is reduced (range 4 to 1 minutes). Litter mate controls injected with the same concentration of iodoacetate, but respiring air, survive approximately 1 hour. Similar results are obtained with fluoride. Infant rats injected with fluoride and respiring air live for more than 1 hour. Litter mate controls, similarly injected with fluoride, but respiring nitrogen, survive approximately 5 minutes.

Determinations were made to ascertain whether the formation of lactic acid was entirely inhibited by the concentrations of iodoacetate used. Averages of the lactic acid contents of the entire body of three groups of animals, each consisting of nine new born rats, indicate that those surviving in nitrogen for more than 50 minutes contain 145 mg. per cent; others injected with iodoacetate before being placed in nitrogen, 35 mg. per cent; finally, the group sacrificed after receiving iodoacetate, 41 mg. per cent.

\* Aided by a grant from Child Neurology Research (Friedsam Foundation).

Though most tissues of the body can obtain energy from the oxidation of carbohydrate and fat, this does not apply to the brain, which can utilize only carbohydrate as a source of energy. These results, therefore, reveal that under anaerobic conditions the brain of new born rats can be maintained for 50 minutes by the oxidative chain mentioned above (Reaction I). On the other hand, aerobically, cerebral metabolism is supported despite the fact that this mechanism is stopped. This affords evidence for an alternate path of glucose oxidation. Studies of the carbohydrate balance of various tissues are in progress.

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## THE ISOLATION OF PHOSPHATIDYL SERINE FROM BRAIN CEPHALIN, AND IDENTIFICATION OF THE SERINE COMPONENT

*Sirs:*

In a recent paper<sup>1</sup> it was shown that cephalin prepared in the usual manner from brain did not contain, as previously accepted, all of its nitrogen in the form of ethanolamine, but that from 40 to 70 per cent was in the form of a  $\beta$ -hydroxyamino acid. From its reactions it appeared probable that this amino acid was serine, but it was not isolated. The cephalin fraction containing the amino acid has now been isolated, and the amino acid crystallized as analytically pure *l*(+)-serine. We shall call this phosphatide "phosphatidyl serine."

*Isolation of Phosphatidyl Serine*—The separation is based on the discovery that phosphatidyl serine is more soluble than the rest of the "cephalin" fraction in a mixture of alcohol and chloroform. Cephalin is dissolved in 15 parts of chloroform, and partially precipitated by addition of an equal volume of absolute alcohol. The supernatant solution contains the greater part of the phosphatidyl serine. By repeating the procedure on material recovered from the supernatant solution, preparations have been obtained which contained 97 per cent of their nitrogen in the form of hydroxyamino acid. The elementary composition of the product, dried at 80° *in vacuo*, was C 60.9, H 9.3, N (Dumas) 1.60, P 3.65,  $\alpha$ -amino acid nitrogen by the ninhydrin-CO<sub>2</sub> reaction<sup>2</sup> 1.55.

*Isolation of Serine*—The phosphatidyl serine was hydrolyzed by boiling in 6 N HCl for 30 hours. The fatty acids were filtered off, the Cl and PO<sub>4</sub> were removed by Ag<sub>2</sub>O, and the dissolved Ag by H<sub>2</sub>S. The clear filtrate was concentrated to a small volume, and treated with 10 volumes of absolute alcohol. The amino acid crystallized overnight in the ice box. It was recrystallized twice

<sup>1</sup> Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).

<sup>2</sup> Van Slyke, D. D., and Dillon, R. T., *Compt.-rend. trav. Lab. Carlsberg*, **121**, 480 (1938).

in the same way. The yield of recrystallized serine was 38 per cent of the theoretical. It contained 1.68 per cent of ash. Analysis, corrected for ash, C 34.28, H 6.57, N (Dumas) 13.2,  $\text{NH}_2\text{-N}^3$  13.16,  $\alpha$ -amino acid N by the ninhydrin- $\text{CO}_2$  method<sup>2</sup> 13.14.

*Rotation*—A solution in 1 N HCl containing 52.6 mg. of the amino acid per cc. showed in a 1 dm. tube a rotation of  $+0.75^\circ$  with yellow light;  $\alpha_D^{20} = +14.2^\circ$ . Fischer and Jacobs<sup>4</sup> give  $+14.5^\circ$ .

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<sup>2</sup> Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).

<sup>4</sup> Fischer, E., and Jacobs, W. A., *Ber. chem. Ges.*, **39**, 2942 (1906).

## A SPECIFIC GROWTH INHIBITION REVERSED BY PANTOTHENIC ACID

*Sirs:*

The recent findings of Woods<sup>1</sup> of the antisulfanilamide effect of *p*-aminobenzoic acid, together with those of McIlwain<sup>2</sup> who showed that pyridine-3-sulfonic acid produced a specific growth inhibition in certain organisms reversed by additional nicotinic acid, suggested that the sulfonic acid analogue of pantothenic acid might prove inhibitory. This compound was prepared by a method similar to that described by Williams *et al.*<sup>3</sup> for preparation of sodium pantothenate.

5 gm. of  $\alpha$ -hydroxy- $\beta$ , $\beta$ -dimethyl- $\gamma$ -butyrolactone were melted; to the melt were added 5.7 gm. of the dry, powdered sodium salt of taurine. The mixture was held at 120° for 5 hours and then cooled. Amino nitrogen determinations (Van Slyke) showed the disappearance of 80 per cent of the free amino nitrogen during the reaction; this was quantitatively liberated by hydrolysis of the reaction product with 0.5 N HCl for 25 minutes at 115°. This indicates the formation in 80 per cent yield of N-( $\alpha$ , $\gamma$ -dihydroxy- $\beta$ , $\beta$ -dimethylbutyryl)-taurine.

The biological activity of the reaction product was determined with *Lactobacillus arabinosus* 17-5 in the base medium described by Snell and Wright<sup>4</sup> to which nicotinic acid was added, and which contained varying amounts of calcium pantothenate. The results are given in the table. Turbidity measurements were made after 24 hours incubation.

In the presence of small amounts of calcium pantothenate, addition of the condensation product inhibited growth; this inhibition was reversed by addition of larger amounts of calcium

<sup>1</sup> Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).

<sup>2</sup> McIlwain, H., *Brit. J. Exp. Path.*, **21**, 136 (1940).

<sup>3</sup> Williams, R. J., Mitchell, H. K., Weinstock, H. H., Jr., and Snell, E. E., *J. Am. Chem. Soc.*, **62**, 1784 (1940).

<sup>4</sup> Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, in press (1941).

Calcium pantothenate	Condensation product (pantothenic acid analogue)	Moist cells	Calcium pantothenate	Condensation product (pantothenic acid analogue)	Moist cells
$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per cc.	$\gamma$ per 10 cc.	$\gamma$ per 10 cc.	mg. per cc.
0	0	0.2	3	1,000	7.5
0	100	0.0	10	1,000	10.2
0.4	0	9.4	3	10,000	0.35
0.4	100	5.8	10	10,000	1.4
0.4	300	1.6	30	10,000	5.0
0.4	1,000	0.5	0.4	3,000*	10.8
0.4	10,000	0.3	0.4	10,000*	12.8

\* To these tubes not the reaction product, but 3000 and 10,000  $\gamma$  each of taurine and lactone were added.

pantothenate. Mixtures of taurine and the lactone (or either separately) had no such inhibitory action; indeed, at these high concentrations, the lactone somewhat stimulates growth. Addition of other B vitamins or of hydrolyzed casein had no effect. Exactly the same relationships hold with yeast as with the above organism, except that somewhat larger quantities of the reaction product are required to produce inhibition.

The phenomenon is being further investigated, and attempts made to isolate in pure form the extremely soluble and hygroscopic sulfonic acid or a suitable derivative.

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## THE OXIDATION OF *p*-AMINOBENZOIC ACID CATALYZED BY PEROXIDASE, AND ITS INHIBITION BY SULFANILAMIDE

*Sirs:*

Woods,<sup>1</sup> in demonstrating the antagonism between *p*-aminobenzoic acid and sulfanilamide, concluded that the inactivation of sulfanilamide by *p*-aminobenzoic acid should be due to competition for an enzyme between the "essential metabolite" (Fildes<sup>2</sup>) and the drug. In the following an enzymatic oxidation of *p*-aminobenzoic acid which is inhibited by sulfanilamide will be described. During experiments conducted for other purposes, it was observed that peroxidase catalyzes the oxidation of *p*-aminobenzoic acid by hydrogen peroxide. The enzyme used was prepared from horseradish according to Elliott.<sup>3</sup> No reaction between *p*-aminobenzoic acid and  $\text{H}_2\text{O}_2$  takes place without the enzyme. When peroxidase is added, a red color develops and  $\text{H}_2\text{O}_2$  disappears in amounts proportional to the color formed. The red dye can be reversibly reduced with sodium hyposulfite. Most probably a rather complex mixture of oxidation and condensation products is formed in this reaction (*cf.* analogous experiments with *p*-toluidine<sup>4</sup>).

The inhibition by sulfanilamide is most easily demonstrated by comparing the red color developed in the absence and in the presence of the drug. Proportionately less  $\text{H}_2\text{O}_2$  disappears in the presence of sulfanilamide. Sulfathiazole is likewise active, and sulfapyridine is even somewhat more active than sulfanilamide.

0.3 mg. of enzyme (= 0.6 purpurogallin unit<sup>5</sup>) in 4 ml. of acetate buffer, pH 6, containing  $2.5 \times 10^{-3}$  mole per liter of *p*-aminobenzoic acid and  $5 \times 10^{-4}$  mole per liter of  $\text{H}_2\text{O}_2$ , is used.

<sup>1</sup> Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).

<sup>2</sup> Fildes, P., *Brit. J. Exp. Path.*, **21**, 67 (1940).

<sup>3</sup> Elliott, K. A. C., *Biochem. J.*, **26**, 1281 (1932).

<sup>4</sup> Saunders, B. C., and Mann, P. J. G., *J. Chem. Soc.*, 769 (1940).

<sup>5</sup> Willstätter, R., and Stoll, A., *Ann. Chem.*, **416**, 21 (1918).



After 5 minutes, the reaction is stopped by the addition of catalase which immediately destroys the remaining  $\text{H}_2\text{O}_2$ . The color is read on a photoelectric colorimeter.

Sulfanilamide, $10^{-3}$ mole per l . . . .	1.5	6.1	13	33
Inhibition, % . . . . .	11	30	51	80

No measurable oxidation of sulfanilamide by the enzyme system could be observed. The peroxidase reaction with other substrates, e.g. pyrogallol and tyramine, was likewise inhibited by sulfanilamide. The inhibition of peroxidase is reminiscent of the inhibition of catalase by sulfanilamide, earlier described by Shinn, Main, and Mellon.<sup>6</sup>

Furthermore it was found that *p*-aminobenzoic acid is oxidized through phenoloxidase in the presence of catalytic amounts of catechol. Potato enzyme was used. A red color develops with oxidation very similar to that formed with peroxidase. This reaction was not inhibited by sulfanilamide.

What connection there may be between the enzymatic reactions described and the biological activity of *p*-aminobenzoic acid and sulfanilamide remains to be decided by further experiments. From earlier work<sup>7</sup> it must be concluded that many organisms acted upon by sulfanilamide are unable to decompose hydrogen peroxide. This makes it difficult to imagine any general interrelation between a peroxidase reaction and sulfanilamide activity. However, connection between the metabolism of phenolic substances and *p*-aminobenzoic acid seems indicated by the action of the latter substance as an anti-gray hair factor, reported by Ansbacher,<sup>8</sup> and by the antagonism between hydroquinone and *p*-aminobenzoic acid with respect to graying, observed by Martin and Ansbacher.<sup>9</sup>

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<sup>6</sup> Shinn, L. W., Main, H. J., and Mellon, R. R., *Proc. Soc. Exp. Biol. and Med.*, **44**, 596 (1940).

<sup>7</sup> MacLeod, C. M., *Proc. Soc. Exp. Biol. and Med.*, **41**, 215 (1939).

<sup>8</sup> Ansbacher, S., *Science*, **93**, 164 (1940).

<sup>9</sup> Martin, G. J., and Ansbacher, S., *J. Biol. Chem.*, **138**, 441 (1941).

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